KAUNAS UNIVERSITY OF TECHNOLOGY

FACULTY OF CHEMICAL TECHNOLOGY

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Processing of elderberry (*Sambucus nigra* L.) pomace into valuable components by conventional, high pressure and ultrasound-assisted extraction techniques

Master's Final Degree Project

Supervisor
Assoc. prof. Vaida Kitrytė

KAUNAS, 2018

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KAUNO TECHNOLOGIJOS UNIVERSITETAS

CHEMINĖS TECHNOLOGIJOS FAKULTETAS

Šeivamedžio (*Sambucus nigra* L.) uogų išspaudų perdirbimas į vertingus komponentus taikant tradicinius, aukšto slėgio ir ultragarso ekstrakcijos metodus

Baigiamasis magistro projektas

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„Processing of elderberry (*Sambucus nigra* L.) pomace into valuable components by conventional, high pressure and ultrasound-assisted extraction techniques“

DECLARATION OF ACADEMIC INTEGRITY

4 June 2018

Kaunas

I confirm that the final project of mine, **Agnė Laurinavičienė**, on the topic „**Processing of elderberry (*Sambucus nigra* L.) pomace into valuable components by conventional, high pressure and ultrasound-assisted extraction techniques**“ is written completely by myself; all the provided data and research results are correct and have been obtained honestly. None of the parts of this thesis have been plagiarised from any printed, Internet-based or otherwise recorded sources. All direct and indirect quotations from external resources are indicated in the list of references. No monetary funds (unless required by law) have been paid to anyone for any contribution to this project.

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Kaunas, 2018. 108 p.

Santrauka

Sambucus nigra L., ūksmininių šeimai priklausantis, lapus metantis krūmas, kuris yra apie 5 m aukščio, subrandinantis tamsias, beveik juodas, uogas, kurios auga kekėmis. *S. nigra*, šeivamedis, yra plačiai paplitęs Europoje ir uogos dažniausiai yra perdirbamos į įvairius produktus, tokius kaip, koncentratai, sirupai, uogienės. Šeivamedis yra žinomas liaudies medicinoje kaip vaistas nuo įvairių uždegiminių ligų.

Daugiausia informacijos galima rasti apie šeivamedžio uogas ir sultis, kai tuo tarpu mokslinių tyrimų, susijusių su išspaudomis, yra mažai. Tačiau, keli autoriai mano, kad išspaudos galėtų būti pigus ir alternatyvus natūralių, biologiškai aktyvių, nepolinių ir polinių junginių, šaltinis kitoms pramonės šakoms, kaip maisto, farmacijos ir kosmetikos.

Šio darbo tikslas taip pat buvo nustatyti, ar šeivamedžio uogų išspaudų perdirbimas gali būti alternatyvus vertingų nepolinių ir polinių komponentų šaltinis. Dėl šios priežasties, buvo pasirinkti tradiciniai ir inovatyvūs ekstrakcijos metodai, tokie kaip, ekstrakcija superkritiniu CO_2 (SKE-CO_2), pagreitinta ekstrakcija tirpikliais ir ultragarso ekstrakcijos įvairių. Inovatyvūs ir tradiciniai ekstrakcijų metodai buvo lyginami tarpusavyje ir nustatoma, ar galima sutrumpinti ekstrakcijos laiką, sumažinti tirpiklių naudojimą ir padidinti vertingų komponentų išeigą.

Tyrimo metu buvo optimizuoti SKE-CO_2 parametrai nepolinių junginių išgavimui iš šeivamedžio išspaudų po sulčių spaudimo, taip pat buvo nustatoma, kokia pakopinė ekstrakcijos schema yra efektyviausia ir nepolinių, ir polinių vertingų komponentų išgavimui, buvo tiriama išgautų nepolinių frakcijų riebalų rūgščių sudėtis, naudojant GS-FID, *in vitro* antioksidacinis aktyvumas (TPC, $ABTS^{\bullet+}$, ORAC, oxipres), nepolinio ekstrakto, išgauto po SKE-CO_2, lakiųjų junginių sudėtis, naudojant GS-MS ir preliminari nepolinių ir polinių frakcijų fitocheminė sudėtis, naudojant UPLC-QTOF-MS.

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Summary

Sambucus nigra L., which belongs to *Adoxaceae* family, is a deciduous shrub that reaches about 5 m in height and produces nearly black berries which are grown in clusters. *S. nigra*, elderberry, is widespread in Europe and it is usually processed into value-added food products, such as concentrates, syrups, jams. In addition, elderberry is known as a traditional remedy for various kinds of ailments. Mostly, *S. nigra* berries and juice are under study, while little information is so far available on elderberry pomace. Though, several authors predicated that *S. nigra* pomace could be a potential cheap and alternative source of natural bioactive non-polar and polar constituents for other, food, pharmaceutical or cosmetic industries.

In this work, we have also attempted to establish to find evidence, which supporting the need to use *S. nigra* pomace for isolation valuable non-polar and polar constituents. For this reason, conventional and innovative, such as supercritical fluid extraction with CO_2 (SFE-CO_2), pressurized-liquid extraction and ultrasound-assisted extraction (UAE), were used for target compounds isolation. Innovative extraction methods were compared with conventional extraction methods, in order to find is it possible to shorten the extraction time, reduced solvents use, and increased target compounds yields.

After extractions were explored and determined optimal SFE-CO_2 parameters for isolation non-polar fraction from elderberry pomace after juice processing and was developed the best multi-step scheme for both, non-polar and polar, fractions isolation, fatty acid composition of non-polar extracts by GS-FID, *in vitro* antioxidant capacity (TPC, $ABTS^{\bullet+}$, ORAC, oxipres); volatile compounds profile of SFE-CO_2 extract at optimal conditions (35 MPa, 53 °C; 105 min) by GS-MS and preliminary phytochemical composition by UPLC-QTOF-MS were determined for target non-polar and polar extracts.

ABREVIATIONS

AAPH 2,2'-Azobis(2-amidinopropane) dihydrochloride;

ABTS$^{\bullet+}$ 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid);

AC Acetone;

ANOVA Analysis of variance;

CCD Central composite design;

DW dry sample weight;

EtOH Ethanol;

GAE Gallic acid equivalent;

HCN Hydrogen cyanide;

HE Hexane;

UPLC Ultra performance liquid chromatography;

ORAC Oxygen radical absorbance capacity;

PLE Pressurized liquid extraction;

QUENCHER Quick, easy, new, cheap and reproducible;

RSM Response surface methodology;

SFE-CO_2 Supercritical carbon dioxide;

SD Standard deviation;

TEAC Trolox equivalent antioxidant capacity;

TPC Total phenolic content

INTRODUCTION

Sambucus nigra L., which belongs to *Adoxaceae* family, is a deciduous shrub that reaches about 5 m in height or rarely small tree growing up to 10 m in height. This plant produces nearly black berries which are grown in clusters. *S. nigra*, elderberry, is widespread in Europe and it is usually processed into value-added food products, such as concentrates, syrups, jams, wine [1]. In addition, elderberry is known as a traditional remedy for various kinds of ailments (cold, coughing, influenza) and is processed into different forms of dietary supplements (syrups, drops, tablets, emulsions or suspensions) [2]. The demand of *S. nigra* products are increasing and since the 1980s, elderberry has been grown on commercial plots all over the world and in 2005 over 11 million hectares of elderberry plots were determined [2, 3].

The one of the most important trends in areas of food science and technology is the growing demand of natural antioxidant compounds and various plant-origin materials, including berry fruits, are unlimited sources of them. In addition, large amounts of berries harvests are processed into longer shelf life food products, such as juice, jams, concentrates. However, berries processing into other products results in high amounts of residues, namely pomace [4]. According to Directive 2008/98/EC, if such residues are not processed into other products they are considered as a waste [5]. Currently, the major portion of berry pomaces are discarded or used as animal feed stock, despite the fact that a considerable amount of valuable nutrients and bioactive phytochemicals are retained in pomace [4, 6, 7]. In addition, FAO noted that the food products supply chain organization, from initial production down to final household consumption, is very important issue in order to reduce the wastage of food. According to EC (2016), food wastage reached 88 million tonnes in 2012, including edible food and inedible parts associated with food and the food wastage was estimated to reach 20% on average, taking into consideration different alimentary sources [3]. Therefore, the valorisation of cheap biomass, such as berry pomaces, could be regarded as one of the strategies to obtain higher added value nutrients and phytochemicals with multipurpose applications in food pharmaceutical and nutraceutical industries. Also, the development of efficient valorisation (biorefining) schemes could offer alternative solutions related to ecological problems of pomace wastage.

Up to date, most part of the literature data focus on *S. nigra* berries and only a few works has been dedicated to elderberry by-product, regardless of its high amount (20-40%) of total berry after juice processing. Seabra et al. (2010) and Fazio et al. (2013) reported that elderberry pomace is a source of anthocyanins (cyanidin 3-glucoside; cyanidin 3-sambubioside), polyunsaturated fatty acids (linolenic, linoleic, oleic acids) and vitamins [2, 8, 9]. Non-polar and polar fractions from elderberry pomace can be isolated using conventional extraction methods,

namely Soxhlet and solid-liquid extraction (SLE), high-pressure extraction methods, such as supercritical fluid extraction with carbon dioxide (SFE-CO_2) and pressurized-liquid extraction (PLE) and using ultrasound-assisted extraction technique. Extraction parameters (time, solvents, pressure), yields of target extracts and their chemical composition are the most important criteria to verify the optimal extraction schemes and to determine the efficiency of each process.

The main aim of the work – to develop multi-step elderberry (*Sambucus nigra* L.) pomace processing schemes for valuable non-polar and polar components isolation by conventional, high pressure and ultrasound-assisted extraction techniques. In order to achieve this aim, the following goals of this work were set:

1. To characterize *S. nigra* pomace after juice processing by determining selected chemical composition (lipid, protein, nitrogen, moisture and dry matter content) and *in vitro* antioxidant activity (total phenolic content and $ABTS^{\bullet+}$ scavenging capacity) parameters.
2. To optimize supercritical carbon dioxide extraction parameters for lipophilic constituent isolation from *S. nigra* pomace.
3. To develop multi-step *S. nigra* pomace valorisation (biorefining) scheme by comparing the efficiencies of conventional, high pressure and ultrasound-assisted extraction techniques for non-polar and polar fraction isolation from *S. nigra* pomace.
4. To evaluate *in vitro* antioxidant potential (total phenolic content, $ABTS^{\bullet+}$ and oxygen radical scavenging (ORAC) capacity) of various *S. nigra* pomace non-polar and polar extracts and solid residues after different steps of extraction and to determine the effects of the selected non-polar and polar *S. nigra* pomace extracts on the oxidative stability of rapeseed oil.
5. To determine and characterize fatty acids and volatile compound profiles of non-polar *S. nigra* extracts by GC-FID and GC-MS analysis.
6. To evaluate phytochemical composition of selected *S. nigra* pomace non-polar and polar extracts by UPLC-QTOF-MS.
7. To determine the major cyanogenic glycoside sambunigrin amounts in *S. nigra* berry fruits, juice, pomace and selected non-polar and polar extracts.

1. LITERATURE REVIEW

1.1. General characteristics of *Sambucus nigra* berry fruits

The plants of *Sambucus* L. are assigned to *Adoxaceae* family, which contains of 7 different genera that have berry fruit. *Sambucus* L. consist of 5–30 species and 6–11 subspecies, depending on the taxonomy system. The most commonly occurring species are *Sambucus nigra* L. (Fig.1), commonly known as elder, elderberry, black elder, European elder, European elderberry and European black elderberry. *Sambucus nigra* L. has 3 subspecies: *S. nigra* L. ssp. nigra, *S. nigra* L. ssp. canadensis, *S. nigra* L. ssp. cerulean [2, 10].

The *Sambucus nigra* L. is a deciduous shrub that reaches 4-6 m in height or seldom small tree growing up to 10 m in height. Elderberry produces nearly black, very dark purple fleshy fruits that grow in clusters. *S. nigra* blooms from May to July and the harvest season occurs from the end of August to September [10, 11]. *S. nigra* L. ssp. canadensis, *S. nigra* L. ssp. cerulean are native to North America and *S. nigra* L. ssp. nigra naturally occurs in most of Europe (except for certain parts of Scandinavia and Russia) and in North Africa (Fig.2) [2].

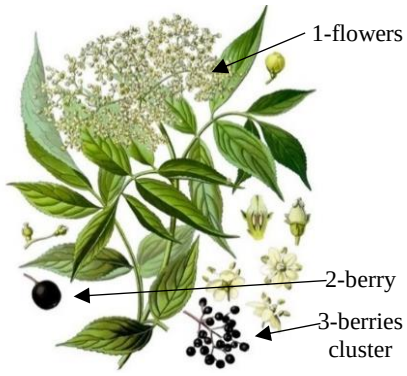


Figure 1. *Sambucus nigra* L. anatomical parts
[Franz Eugen Köhler, Köhler's Medizinal-Pflanzen, 1897 (modified)]

Figure 2. *Sambucus nigra* L. distribution map* [1]
*from Internet source: https://www.cabi.org/isc/datasheet/48259

In many countries, large quantities of berry fruits are collected from wild plants. However, since the 1980s, elderberry has been grown on experimental and commercial plots (Table 1).

Table 1. Worldwide collection area and harvested quantities of *Sambucus nigra* L. in 2005 [12]

Scientific name	Common name	Area (ha)	Quantity (t)
Sambucus nigra L. flowers	Elder tree, flowers	1,818,403	19
Sambucus nigra L. leaves	Elder tree, leaves	1,766,722	6
Sambucus nigra L. berries	Elder tree, berries	11,387,553	472

The *Sambucus nigra* L. yield depends on the growing site, condition, cultivar, and can vary from 1 kg/bush for wild-harvested genotypes to 23 kg/bush for some cultivars [2]. *S. nigra* are not widely grown in Lithuania and as an ornamental medicinal shrub grown in gardens, parks

and elderberry is self-spreading and sometimes found in forests. While in the world the biggest *S. nigra* producers are located in Germany, Austria, Denmark, Poland, Italy, and the Czech Republic. According to the German Federal Statistical Office, the production of elderberry fruit amounted to 1576 tonnes in 2013, 1759 tonnes in 2015 and to 1979 tonnes in 2017. In Austria, the total crop of elderberry fruit varied between 6949 and 10100 tonnes between 2016 and 2017 [2]. In the last two decades the *S. nigra* plantation has increased significantly in Portugal, being mainly cultivated in the northern part and producing annually between 1500 and 2000 tonnes of *S. nigra* berries [13].

1.2. *Sambucus nigra* L. products

Various anatomical parts of *S. nigra* plant are used in food, pharmaceutical and cosmetic industries, while the major anatomical part is berries (Fig.3) [14]. Major portion of elderberries are frozen directly after harvesting period or the fresh berries can be pressed and the resulting juice frozen [7]. *S. nigra* berries are mainly processed into food products, such as concentrates, jelly, jam, ice cream, colorants or syrups (Table 2). These berries are also used in the preparation of pies, punch and liqueurs [15, 16]. Among elderberry products, juice and wine are most widely marketable [1].

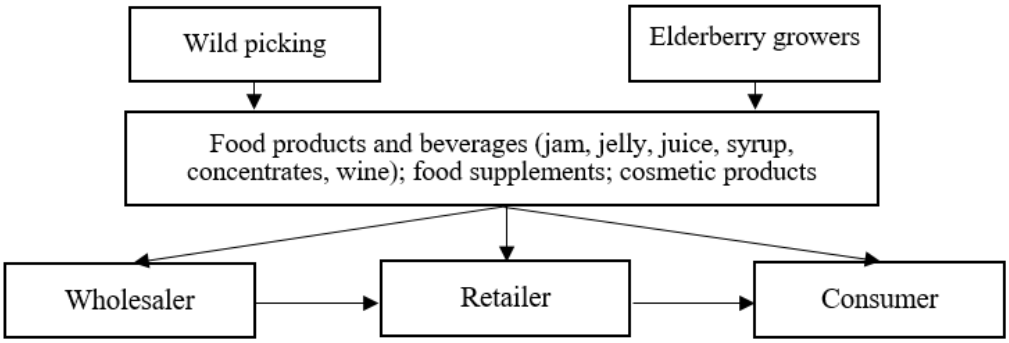


Figure 3. *S. nigra* value chain [16]

S. nigra flowers, characterized by their pleasant, characteristic aroma, currently known as elderflower aroma, are mainly used as flavouring ingredient to produce beverages [17]. In traditional medicine of various countries, tea from *S. nigra* flowers is used against colds, flu and fewer [16]. *S. nigra* flowers are classified as a medicinal product according to European Medicines Agency and elderberry fruit juice or concentrates are marketed as nutraceuticals in the world [16, 18]. According The Commission of the European Communities, *S. nigra* berry extract and *S. nigra* flower water can be used for skin conditioning and refreshing (Table 3).

Table 2. *S. nigra* food products and beverages

Company*, country	Product example*	Product composition*
Premium Rosa, Poland	Elderberry juice	100% juice from fresh elderberries
	Freeze-dried elderberry powder	100% powder made from elderberries
	Juice from apples and elderflower	Juice from fresh apples and elderberries
	Low-sugar black elderberry syrup	Elderberry juice
	Elderflower syrup	Elderflower
Fort Wayne, Indiana	Standardized 3.2% Liquid	Concentrate is produced via a physical process
	Standardized 6.5% Powder	Powder that is extracted via a solvent-free physical process
	Elderberry Juice Powder	Spray-dried *S. nigra* berries solids
	Elderberry Juice Concentrate	Elderberry juice
Norm's Farms, USA	Elderflower syrup	Elderflowers et al.
	Elderflower ginger syrup	Organic elderflowers et al.
	Elderberry jam	*S. nigra* berries
	Elderberry extract	*S. nigra* berries
	Elderberry wellness syrup	*S. nigra* berries
	Elderberry ginger pecan jam	*S. nigra* berries
	Elderberry jelly	Elderberry juice et al.
	Children's formula	Elderberries extract et al.
Runoland, Poland	Mousse of elderberry	*S. nigra* berries
Sad Danków, Poland	Elderberry jam	*S. nigra* berries
BioAvena, Poland	Elderberry juice	100% elderberry juice
Products with Tradition, Poland	Black elderberry vinegar	Elderberry flowers (50%)
Biopurus, Germany	Cold pressed elderberry seed oil	*S. nigra* berries
Coca-cola, Switzerland	Fanta Shokata	Beverage with elderflower aroma
Swanson Health Products Europe	Natural elderberry extract	Berries and flowers
Obst Trautner GMBH, Germany	Fresh elderberries	Fresh, freeze-dried elderberries
Monin, France	Elderflower Monin syrup	Syrup with elderflower extract
Łowicz, Poland	Elderberry jam	*S. nigra* berries
Gourmet Berner, Germany	"Hugo" elderflower liqueur	Elderflower extract
The Amber Brewery, Poland	Amber Czarny Bez	Beer with elderflowers
Euro-wino, Poland	Wine from elderberry	*S. nigra* berries
Gourmet Wales, UK	Elderberry Wine	Ripe *S. nigra* berries
Wyldewood Cellars, USA	Elderberry Wine	Ripe *S. nigra* berries
Lebkuchen-Schmidt GmbH, Germany	Elderberry Wine	Ripe *S. nigra* berries
Manischewitz Winery, USA	Elderberry Wine Kosher	Ripe *S. nigra* berries
Lyme Bay Winery, UK	Elderberry Wine (Silver 2013 from The Taste of the West Awards)	Ripe *S. nigra* berries

*Companies, product examples and product compositions, which were noted in Table, are from Internet source. Information of elderberry products was noted only from official companies' websites.

According to the Food and Agriculture Organization (FAO) data, about one-third of all food produced for human consumption in the world is lost or wasted, while 54% are lost in production steps, postharvest handling and storage, and other 46% are caused by processing, distribution and consumption (in weight basis about 40–50% of fruits and vegetables, 20% of oilseeds are lost) [19, 20].

Table 3. *S. nigra* pharmaceutical and cosmetics products

Company*, country	Product example*	Product composition*
	Nutraceuticals	
Gaia Herbs, USA	Black Elderberry	Fruit extract
	Black Elderberry NightTime Syrup	Fruit juice concentrate
	GaiaKids® Black Elderberry Syrup	Fruit juice concentrate
	GaiaKids® KidsDefense Herbal Drops	Flowers
	GaiaKids® Sniffle Support Herbal Drops	Fruits and flowers
	Vitex Elixir for Women	Fruit juice concentrate
Sambucol, UK	Sambucol Immuno Forte	Fruit juice
	Sambucol for Kids	Fruit juice
	Sambucol Immuno Forte Pastilles	Fruit juice
Nature`s way, USA	Original Sambucus	Fruit extract
	Sambucus Immune Syrup	Fruit extract
	Sambucus FluCare	Fruit extract
Now foods, USA	Elderberry 500 mg Veg Capsules	Fruit juice concentrate
	Cosmetics	
Refan Ltd, Bulgaria	Body cream	Elder fruit extract
	Body lotion	Elder fruit extract
	Hand cream	Elder fruit extract
	Sugar body scrub	Elder fruit extract
	Shower gel	Elder fruit extract
	Bath salts	Elder fruit extract
	Deo roll on	Elder fruit extract
GG's True Organics, Austria	Gentle Cleansing Foam	Elderflower extract
	Balancing Tonic	Elderflower extract
	Intense Moisture Cream	Elderberry seed oil, elderflower extract
	Rich Moisture Cream	Elderberry seed oil, elderflower extract
	Eye Contour Cream	Elderberry seed oil, elderflower extract
	Deep Moisturizing Mask	Elderberry seed oil, elderflower extract
Esent, Poland	Black elderberry peel	Dried and ground elder
Biokosma, Switzerland	Volume& shine hair care product	Elderflower extract

The production of fresh *S. nigra* berries generates a large amount of by-products, while branches are the most abundant and accounts in up to 10% of the total elderberry production [13]. In addition, *S. nigra* juice processing also generates large amounts of pomaces, up to 25% of total berry weight, which are usually used as animal feed, fertilizer or thrown away [21]. However, elderberry pomace after juice processing could be a cheap and alternative source of some valuable constituents for other, cosmetics and pharmaceuticals, industries [13].

Further, not only branches, pomace, but also elderberry seeds are by-products of the beverage, especially winery, industries. According Dulf et al. (2013) elderberry seed residues could be used as valuable material to isolate non-conventional seed lipophilic fraction with unique chemical properties and these non-polar fraction could be widely used in the healthcare industry [22].

1.3. Chemical composition of *Sambucus nigra* L. berry fruits and their products

1.3.1. Macro nutrients

The chemical composition of *S. nigra* depends on many factors, such as genotype, degree of ripeness and environmental conditions. The traditional quality parameters in fruits include dry matter, sugars, dietary fiber and organic acids [23]. Chemical composition of *S. nigra* fruits is presented in Table 4. The carbohydrate content of *S. nigra* berries amounts to ~18% and 7% of which is dietary fiber. Fiber fraction includes pectin, pectic acid (polygalacturonic acid), protopectin (hemicellulose) and calcium pectinate [24]. The total sugar content in fruits varies from 68 g/kg to 104 g/kg fresh weight, while the most abundant sugars are fructose (34-52 g/kg FW) and glucose (42-50 g/kg FW), while sucrose was determined only in trace amounts (0.5-1.7 g/kg FW) [25]. The most abundant organic acids found in elderberries are citric (3-5 g/kg FW), malic (1.0-1.3 g/kg FW), shikimic (0.1-1.0 g/kg FW) and fumaric acids (0.1-0.3 g/kg FW). Protein content in elderberry fruits and leaves is ~3%, in flowers ~2%. Amino acids in fruits, flowers and leaves occur in the free or conjugated form [24], while glutamic, asparagic acid and alanine are the dominant amino acids [2]. The content of ascorbic acid ranges between 6 and 25 mg/100 g of *S. nigra* fruit and depends on the cultivar. According to Barros et al. (2011) research, ascorbic acid content in wild *S. nigra* is 13–35 mg/100 g and 28–34 mg/100 g, depending on the growing location [26].

Table 4. Chemical composition of *S. nigra* fruits [27]

Chemical parameter	Amount	Amino acids	Amount, g/100 g
Dry matter, g/100 g	20.2	Lysine	0.09
Total sugars, g/100 g	8.9	Alanine	0.24
Reducing sugars, g/100 g	8.6	Threonine	0.07
Cellulose, g/100 g	1.7	Glycine	0.07
Pectin, g/100 g	0.2	Valine	0.17
Pectin acid, g/100 g	0.2	Serine	0.17
Protopectin, g/100 g	0.04	Proline	0.09
Ca-pectate, g/100 g	1.5	Leucine	0.21
Total acidity, % citric acid	1.3	Isoleucine	0.09
Ash, g/100 g	0.9	Methionine	0.03
K, mg/100 g	391.3	Histidine	0.06
P, mg/100 g	54.0	Phenylalanine	0.12
Ca, mg/100 g	28.1	Glutamine	0.31
Na, mg/100 g	217.0	Asparagine	0.30
Mg, mg/100 g	26.0	Cysteine	0.01
Fe, mg/100 g	1.9	Tyrosine	0.20

S. nigra seed oil contains α-tocopherol and γ-tocopherol (0.5 and 2.6 μg/g oil, respectively) [9], while the residues after oil pressing contain α-, β-, γ- and δ-tocopherols up to 12 mg/100 g and α-tocotrienol (0.9 mg/100 g) [26]. Lipids are accumulated mostly in *S. nigra* seeds (~22.4%).

The major fatty acids are polyunsaturated fatty acids (Table 5), which constitute 75% and 22 % of total fatty acids in seeds and seed flour, respectively. Polyunsaturated fatty acids that are present in highest concentrations in seeds are linolenic, linoleic and oleic acids [2].

Table 5. Fatty acids (% of total fatty acids) composition of wild *S. nigra* seeds [20]

Species ***S. nigra***	**SFA***	**MUFA***	**PUFA***	**VLCSFA***	**n-6/n-3***	**PUFA/SFA***
TLs total lipids	11	14	75	0.3	0.8	7
PLs polar lipids	30	18	51	4	4	2
MAGs monoacylglycerols	13	11	76	–	2	6
DAGs diacylglycerols	20	22	58	1	5	3
FFAs free fatty acids	38	9	53	0.3	1	1
TAGs triacylglycerols	9	12	78	0.06	1	9
SEs sterol esters	35	30	35	3	2	1

*: SFA saturated fatty acids; MUFA monounsaturated fatty acids; PUFA polyunsaturated fatty acids; VLCSFA very long chain saturated fatty acids. n-6/n-3: omega-6 and omega-3 fatty acids ratio.

Linoleic acid is a doubly unsaturated fatty acid, also known as an omega-6 fatty acid, occurring widely in plant glycosides and an essential fatty acid in human nutrition because it cannot be synthesized by humans. Linoleic acid is an important ingredient in the skin, as a barrier to water penetration. Further, this fatty acid produces arachidonic acid, a precursor of bioactive metabolites called eicosanoids (prostaglandins, thromboxane A2, prostacyclin I2, leukotriene B4, anandamide), which regulates a large number of physiological processes [28–30].

Linolenic acid is also a component of cell membranes and is converted to the longer chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA have been associated with a number of health benefits. For example, in regard to cardiac health, these omega-3 fatty acids have been shown to protect against coronary heart disease, sudden cardiac death and heart failure, possibly through anti-arrhythmic, anti-thrombotic, anti-atherosclerotic and anti-inflammatory mechanisms [31].

1.3.2. Bioactive phenolic compounds

Phenols are compounds having one or more aromatic rings with one or more hydroxyl groups and are generally classified as phenolic acids, flavonoids, stilbenes, coumarins and tannins. Phenolics are products of the secondary metabolism in plants that provides essential reproductive and growth functions of plants, for example, acting as protective mechanisms against pathogens, parasites and predators, as well as contributing to plant colour [32]. Classification of phenolic phytochemicals is presented in Figure 4.

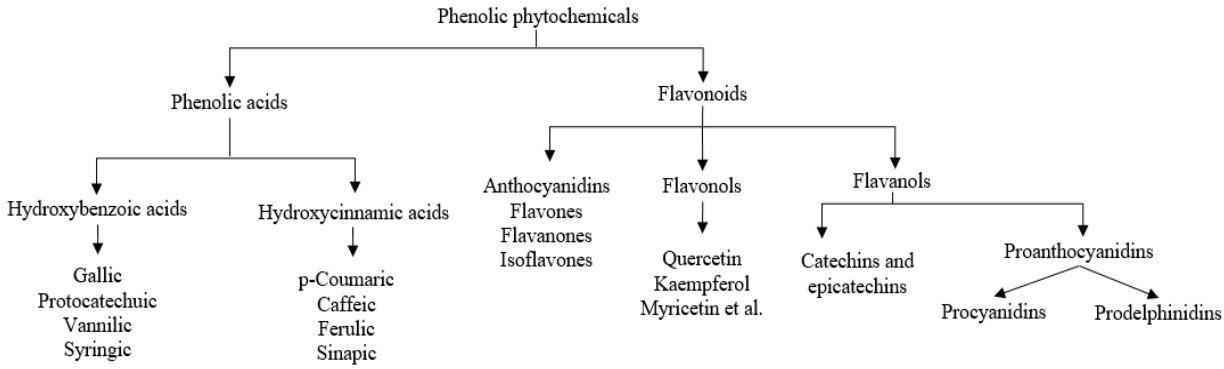
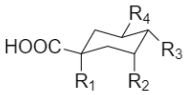


Figure 4. Classification of phenolic phytochemicals [32]

Phenolic acids can be subdivided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxycinnamic acid derivatives include *p*-coumaric, caffeic, ferulic, and sinapic acids [24, 32]. Structures of hydroxycinnamic acids which are found in *S. nigra* are shown in Table 6 and the content of phenolic acids in fruits, juice, liqueur, tea and spread are given in Table 9. The amount of hydroxycinnamic acids was in the range of 15-46 mg/kg, while the highest amount of was determined in berries and the lowest – in elderberry juice.

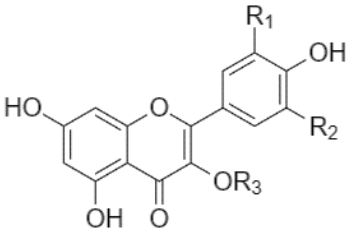
Table 6. Chemical structures of hydroxycinnamic acids in *S. nigra* [24]



Phenolic acids	R_1	R_2	R_3	R_4
3-*O*-Caffeoylquinic acid	OH	Caffeic acid	OH	OH
4-*O*-Caffeoylquinic acid	OH	OH	Caffeic acid	OH
5-*O*-Caffeoylquinic acid	OH	OH	OH	Caffeic acid
1,5-Di-*O*-caffeoylquinic acid	Caffeic acid	OH	OH	Caffeic acid
3,5-Di-*O*-caffeoylquinic acid	OH	Caffeic acid	OH	Caffeic acid
3,4-Di-*O*-caffeoylquinic acid	OH	Caffeic acid	Caffeic acid	OH
4,5-Di-*O*-caffeoylquinic acid	H	OH	Caffeic acid	Caffeic acid
3-*O*-*p* -Coumaroylquinic acid	H	*p*-Coumaric acid	OH	OH
5-*O*-*p* -Coumaroylquinic acid	OH	OH	OH	*p*-Coumaric acid

Structures of flavonols which are found in *S. nigra* are shown in Table 7.

Table 7. Chemical structures of flavonols in *S. nigra* [24]

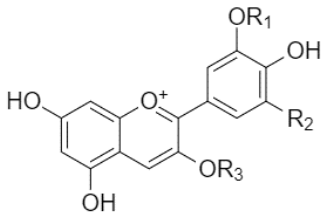


Flavonols	R_1	R_2	R_3
Quercetin 3-*O*-rutinoside	OH	H	Rutinose
Quercetin 3-*O*-galactoside	OH	H	Galactose
Quercetin 3-*O*-vicianoside	OH	H	Vicianose
Quercetin 3-*O*-glucoside	OH	H	Glucose
Quercetin 3-*O*-(6"-acetyl) galactoside	OH	H	6-acetyl galactose
Quercetin 3-*O*-(6"-acetyl) glucoside	OH	H	6-acetyl glucose
Kaempferol 3-*O*-rutinoside	H	H	Rutinose
Kaempferol 3-*O*-glucoside	H	H	Rutinose
Isorhamnetin 3-*O*-rutinoside	OCH_3	H	Rutinose
Isorhamnetin 3-*O*-glucoside	OCH_3	H	Glucose
Myricetin 3-*O*-rutinoside	OH	OH	Rutinose

S. nigra is also known for their content of flavonols, such as quercetin, kaempferol and myricetin, as well as their derivatives (primarily glycosides), which may provide health benefits as dietary antioxidants [33]. According to Table 9, the amount of flavonols in elderberry products was in the range of 3-122 mg/kg, while the highest amount was determined in berries, the lowest – in elderberry juice and even 99 mg/kg was determined in elderberry spread.

Anthocyanins (class of flavonoids) belong to a group of plant constituents, which occur as glycosides and are important plant pigments, responsible for the red, violet and blue colours of plants [15]. Structures of anthocyanins, which are found in elderberry, are shown in Table 8. According to Table 9, the amount of anthocyanins in elderberry products was in the range of 78-733 mg/kg, while the highest amount was determined in berries, the lowest – in elderberry tea and even 238 mg/kg was determined in elderberry juice.

Table 8. Chemical structures of anthocyanins in *S. nigra* [24]



Anthocyanins	R_1	R_2	R_3
Cyanidin 3-glucoside	OH	H	Glucose
Cyanidin 3-sambubioside	OH	H	Sambubiose
Cyanidin 3-sambubioside-5-glucoside	OH	H	Sambubiose
Cyanidin 3,5-diglucoside	OH	H	Glucose
Cyanidin 3-rutinoside	OH	H	Rutinose
Pelargonidin 3-glucoside	OH	H	Glucose
Pelargonidin 3-sambubioside	H	H	Sambubiose
Delphinidin 3-rutinoside	OH	OH	Rutinose

Among cyanidin glycosides the fruit of *S. nigra* mainly contains cyanidin-3-glucoside and cyanidin-3-sambubioside. Two other (minor) anthocyanins are cyanidin-3,5-diglucoside, and cyanidin-3-sambubioside-5-glucoside. In addition, trace quantities of cyanidin-3-rutinoside, pelargonidin-3 glucoside and delphinidin-3-rutinoside are identified in the berries and their products [2]. For example, the most relevant cyanidin 3-*O*-sambubioside amounts ~100 mg/kg in liqueur, ~77 mg/kg in spread, ~48 mg/kg in juice and ~21 mg/kg in tea, while cyanidin-3-*O*-glucoside is detected in values of 88 mg/kg in liqueur, 42 mg/kg in juice and 35 mg/kg in tea) (Table 9).

Polyphenols and anthocyanins amount also depends the growing season and environmental conditions. It was reported that the total anthocyanin content in 2005 was up to 2-fold higher (343 mg of cyanidin-3-glucoside/100 g) than in 2004 (176 mg of cyanidin-3-glucoside/100 g) [2].

Schmitzer et al. (2010) reported that elderberries processing into alcoholic beverages changed the content of phenolic compounds. The amount of polyphenols, such as neochlorogenic acid, chlorogenic acid, quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-rutinoside, cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside,

cyanidin-3-glucoside or cyanidin-3-rutinoside, was higher determined in wine than in must, except cyanidin-3-sambubioside, which decreased during fermentation [34].

Table 9. The content of phenolic compounds in different *S. nigra* products (mg/kg) [35]

Hydroxycinnamic acids	**Control**	**Juice**	**Liqueur**	**Tea**	**Spread**
Chlorogenic acid (trans-5-caffeoylquinic acid)	12.6	0.63	4.8	7	1.6
Neochlorogenic acid (3-caffeoylquinic acid)	9.7	6.8	12.2	4.6	8.2
p-Coumaric acid	3.47	6.66	1.57	-	-
p-Coumaric acid hexoside	-	-	-	16.6	3.6
3-p-Coumaoylquinic acid	10.5	0.16	6.3	-	0.2
4-p-Coumaoylquinic acid	0.81	0.35	0.49	-	0.53
5-p-Coumaoylquinic acid	0.4	0.09	0.13	2.1	0.53
4-Caffeoylquinic acid	0.22	1.2	0.27	1.1	2.2
cis-5-Caffeoylquinic acid	1.4	0.84	0.75	1.2	2.7
Total hydroxycinnamic acids	**46**	**15.4**	**30.1**	**38.7**	**22.4**
Flavanols					
(+) Catechin	22.1	1.5	10.3	-	4.7
(-) Epicatechin	18.0	2.3	16.4	-	23.3
Total flavanols	**40.1**	**3.8**	**26.7**	**-**	**27.5**
Flavonols					
Quercetin-3-O-glucoside	13.6	0.17	5.1	3.7	9.6
Quercetin-3-O-rutinoside	87.6	2.2	38.0	27.3	65.0
Quercetin-acetylglucoside	3.4	0.05	0.19	-	1.9
Quercetin-hexoside pentoside 1	2.8	0.12	0.98	0.62	10.7
Quercetin-hexoside pentoside 2	2.4	0.12	1.25	-	3.0
Kaempferol-3-rutinoside	9.7	0.1	3.8	1.3	7.2
Isorhamnetin-3-rutinoside	3.0	-	1	-	2.0
Total flavonols	**122.2**	**2.8**	**50.5**	**32.8**	**99.4**
Flavanones					
Naringenin hexoside 1	0.2	-	0.03	-	-
Naringenin hexoside 2	0.29	-	0.07	-	0.4
Total flavanones	**0.48**	**-**	**0.08**	**-**	**0.4**
Anthocyanins					
Cyanidin-3-O-glucoside	288.7	42	88.3	5.3	34.8
Cyanidin-3-O-rutinoside	33.3	4.8	10.2	1.2	6.5
Cyanidin-3-O-sambubioside	328.1	47.8	100.4	21	76.8
Cyanidin-3,5-O-diglucoside	19.2	0.1	1.1	0.86	4.7
Cyanidin-3-sambubiosyl-5-glucoside	101	3.5	34.4	28.7	46.6
Total anthocyanins	**732.9**	**98.2**	**237.5**	**78**	**195.5**

Seabra et al. (2010) one of the research goal was to determined anthocyanin content into elderberry pomace and they reported that the major anthocyanin was cyanidin-3-glucoside (14–78 mg/g dry weight) and the sum of this compound and cyanidin-3-sambubioside (15–61 mg/g DW) represents approximately 90% of the total anthocyanin content, as compared to the ranges of 39-153 mg/g DW. Evaluating the anthocyanin content, was noticed that it depending on the extraction method [8].

Plants are a source of many substances affecting functioning of the human organism and its well-being. Antioxidant activity is one of the most desirable properties of natural compounds. Polyphenolic compounds are group of phytochemicals demonstrating particular antiradical properties and a wide range of pharmacological activities like anti-inflammatory [36], immunomodulatory [37, 38], neuroprotective, cardioprotective, antiviral, anticancer [39, 40] and

antimicrobial [41]. Moreover, polyphenols are effective natural food preservatives, preventing oxidative deterioration and microbial contamination [42]. *S. nigra* is a good source of phenolic compounds and can modulate reactive oxygen species (ROS) production and concentration in the organism and it may be useful for the prevention of oxidative stress-related diseases [43]. Elderberry fruits are traditionally used because of their diuretic and diaphoretic properties, and in the treatment of viral infections. Many studies confirmed the benefits of *S. nigra* as an antiviral drug [44–46], also in the treatment and prevention of diabetes, cardiovascular diseases and cancers [47–49]. Human diseases, such as neurodegenerative disorders, chronic asthma, rheumatoid arthritis, allergy, multiple sclerosis, cardiovascular diseases, metabolic syndrome could develop, when human organism suffer from inflammation. So, the main goal of Olejnik et al. (2015) research was to investigate anti-inflammatory effect of gastrointestinal digested elderberry fruit extract and obtained results indicate that elderberry extract reached the small intestine partitions in a modified, but active form and had the ability to weaken the inflammatory response in macrophages. Elderberry also reduced enhanced generation of ROS, recognized as key molecules that play a significant role in the development of inflammatory sicknesses. In addition, Olejnik et al. (2015) noted that future researches, based on cell-based model systems, are needed in order to investigate the effective dose of elderberry extract against inflammatory diseases [37].

1.3.3. Cyanogenic glycosides in Sambucus nigra L.

In addition to health beneficial compounds most *Sambucus* species also contain cyanogenic glycosides, which can be toxic for humans and herbivores if ingested in higher amounts [50]. The most abundant cyanogenic glycoside, that is found in elderberry, is sambunigrin (Fig. 5).

Furthermore, elderberry contains m-hydroxysubstituted glycosides, such as zierin and holocalin [24]. Temperature is possibly critical factor which affect the amount of cyanogenic glycosides. The highest content of cyanogenic glycosides was measured during the spring months and the amount of cyanogenic glycosides decreased in August (temperature < 15 ˚C) and cyanogenic glycosides amount again has increased during autumn (September to October) [35].

Senica et al. (2016) reported that the content of sambunigrin in *S. nigra* changes depending on the growing altitude. The changes of cyanogenic glycosides content in different plant parts is a reaction of single plant to various environmental factors concerned to altitude or genetic adaptation of the elderberry population, which is growing at certain environments. It was observed that presence of radicals increase of cyanogenic glycosides synthesis in *S. nigra* is during the periods of low temperatures and slow vegetative growth. A similar defensive strategy

can be noticed in plants at higher altitudes and shorter lengths of vegetation periods, as they quickly produce more toxins for protection [35].

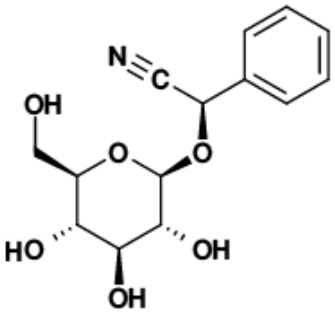


Figure 5. Chemical structure of the major cyanogenic glycoside sambunigrin in elderberry (IUPAC name: (2S)-2-phenyl-2-[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy6(hydroxymethyl)oxan-2-yl]oxyacetonitrile)

These compounds are potentially toxic and life-threatening because they can be hydrolysed resulting in the release of hydrogen cyanide (HCN) (Fig.6). In humans' organism, HCN can be detoxified in three ways. The first way is the conversion in the liver of cyanide to thiocyanate by rhodanese (thiosulphate-cyanide sulphur transferase). The second way is detoxification by direct chemical combination of cyanide with sulphur in the form of an amino acid (di-cysteine) with the formation of 2-aminothiazoline-4-carboxylic acid and cysteine. The third way is the combination of cyanide with hydroxycobalamin (*in vivo* or as a therapeutic expedient) to form cyanocobalamin (vitamin B12) [51].

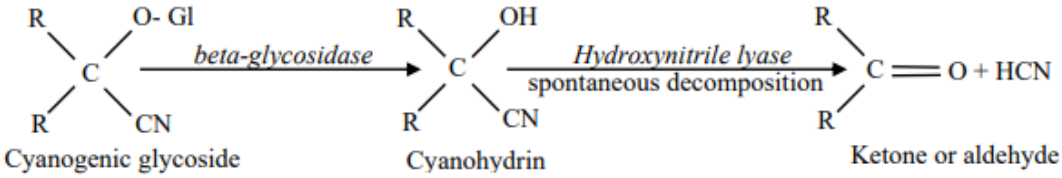


Figure 6. Structure of common plant-derived cyanoglycosides and principle pathway of HCN formation [51]

Cyanogenic glycoside sambunigrin was found in *S. nigra* products, like in juice (10.6 mg/kg), liqueur (0.8 mg/kg), tea (3.8 mg/kg) and spread (0.8 mg/kg), while in the berries was found 18.8 mg/kg [35]. Młynarczyk et al. (2018) reported that the highest amount of sambunigrin was presented in leaves (0.03–0.21 mg/g FW), lower amounts – in flowers (0.00123–0.019 mg/g FW), whereas berries contain the lowest amounts of sambunigrin (0.08–0.77 µg/g FW) [2].

1.4. Isolation and characterization of *Sambucus nigra* L. berry fruit volatile constituents

Due to the growing interest for industrial use of *S. nigra* products for different purposes there is an increasing demand for *S. nigra* cultivars with special quality characteristics including flavor and odour. The taste of elderberries is associated to the content of sugars and acids and the odour is strongly related to the content of volatile compounds [52]. *S. nigra* fruit include essential oils (about 0.01% in fruit), consisting of about 53 volatile compounds [2].

The first goal before the determination and quantification volatile compounds in GC analysis, is the challenge to reduce analysis time. This is represented by the development of miniaturized pre-concentration steps that can be put on-line with the GC instrument, such as sorbent-filled programmed temperature vaporization (PTV) injectors and solid phase micro-extraction (SPME) [53]. SPME was introduced in the early 1990s as a simple and effective adsorption/absorption (based on the used solid/liquid coating) and desorption technique, which eliminates usage of solvents [45]. Solid-phase microextraction (SPME), is a solid phase extraction technique that involves the use of a fiber coated with an extracting phase, that can be a liquid (polymer) or a solid (sorbent), which extracts different kinds of analytes from different kinds of media, that can be in liquid or gas phase (Fig.7). The quantity of analyte extracted by the fibre is proportional to its concentration in the sample as long as equilibrium is reached or, in case of short time pre-equilibrium, with help of convection or agitation [54].

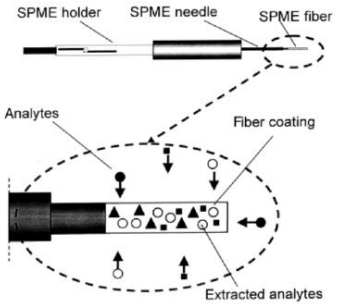


Figure 7. SPME device (fiber coating is exposed to airborne analytes) [55]

Vítová et al. (2013) used sixteen cultivars of *S. nigra* grown in Czech Republic: Albida, Allesö, Aurea, Bohatka, Dana, Haschberg, Korsör, Mammut, Pregarten, Riese aus Voßloch, Sambo, Sampo, Sambu, Samdal, Samyl and Weihenstephan for isolation and characterization of volatile *S. nigra* constituents profile. *S. nigra* content of volatile constituents' chemical groups varied depending on the cultivar. Alcohols content can differ from even 1.2 mg/kg in *Dana* fruits to 3855 mg/kg in *Albida* fruits cultivar; aldehydes content could be from 0.1 mg/kg in *Dana* fruits to 44.3 mg/kg in wild elderberries; esters quantity varied 0.1-73.1 mg/kg.

In 2005, Kaack and co-workers studied even 101 samples of *S. nigra* mature berries, picked in Austria, England and Denmark. In this study, dynamic headspace sampling technique

was applied for collection of volatiles and gas chromatography-mass spectrometry was used for identification of headspace volatiles (Table 10) [52].

Table 10. Volatile compounds isolated from samples of *S. nigra* juice by HD-GS-MS [56]

Compound	Odour	Yield ng/ml	Compound	Odour	Yield ng/ml
Pentanal	fruity, vanilla	73	(Z)-3-hexen-1-ol	fresh green grass	145
ethyl 2-methylbutyrate	fruity	2	nonanal	fruity, elderberry	80
ethyl isovalerate	fruity	29	methyl octanoate	fruity, green	–
hexanal	green, grassy, fruity	328	(E)-2-hexen-1-ol	green pepper	45
2-methyl-1-propanol	fruity	165	(E)-2-octenal	green apple	4
b-pinene	woody	0.2	2-heptylfuran	nutty, roasted	2
isopentyl acetate	fruity	0.4	ethyl octanoate	fruity, floral	14
3-carene	fruity, citrus	3.1	1-octen-3-ol	mushroom	13.0
1-penten-3-ol	mushroom	42	1-heptanol	green, woody	17
myrcene	fruits, citrus, hops	0.1	nerol oxide	floral, elder flower	2
heptanal	fruity, fatty, green	38	methyl nonanoate	fruity, nutty, wine	8
methyl hexanoate	fruity, pineapple	28	decanal	floral, citrus	12
limonene	fruity, orange	2	dihydroedulan	fruity, elderberry	11
2-; 3-methyl-1-butanol	fruity, sweet	614	benzaldehyde	sweet, candy	106
(E)-2-hexenal	fruity, green, apple	56	ethyl nonanoate	fruity, nutty, fatty	8
2-pentylfuran	fruity	21	linalool	flowery, freesia	17
ethyl hexanoate	banana, pineapple	36	1-octanol	fruity, citrus	1
1-pentanol	fruity	–	hotrienol	flowery	2
3-hydroxy-2-butanone	buttery, creamy	17	methyl benzoate	fruity	–
terpinolene	fruity, citrus, pine	1.7	phenylacetaldehyde	floral, hyacinth	9
octanal	fruity, citrus	64	a-humulene	floral, woody	3
methyl heptanoate	fruity, berry	3	a-terpineol	flowery, sweet	0.6
1-octen-3-one	mushroom	0.7	methyl salicylate	minty, sweet	0.2
6-methyl-5-hepten-2-one	fruity, green, sweet	12	ethyl phenylacetate	sweet, honey	3
ethyl heptanoate	fruity, wine	–	2-phenylethyl acetate	fruity, flowery	–
cis-rose oxide	elderflower	3	b-damascenone	fruity, elderberry	4
1-hexanol	green, woody, fruity	145	benzyl alcohol	floral, rose	28
trans-rose oxide	elderflower	0.3	2-phenylethyl alcohol	floral, rose	38
(E)-3-hexen-1-ol	fresh green grass	5	eugenol	spicy, clove	–

From these studies it is noticed that the floral green and floral odours are related to hotrienol, linalool, and other terpenoids, while the characteristic elderberry odour appears to be related with especially *b*-damascenone. Creamy, oily or buttery odour seems to be associated with carboxylic acids and ketones and fruity odours to esters of lower carboxylic acids and alcohols. The fresh green odour appears to be associated with the occurrence of common volatile alcohols and aldehydes with green notes such as 1-hexanol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, hexanal and (*E*)-2-hexenal, respectively [56, 57]. Volatile aldehydes and alcohols, the key compounds in the fresh and green sensorial notes of vegetables and fruits, formed by plants in response to various stresses, play a major role in plant protection mechanisms [58], [59].

1.5. Isolation of valuable non-polar and polar *Sambucus nigra* L. constituents

1.5.1. Conventional extraction methods

Fruits, vegetables and different food products are complex mixtures of bioactive non-polar and polar constituents. Before various components can be analyzed, they must be extracted from the sample matrix. Different extraction methods can be used for this aim, which can be divided into conventional and non-conventional high pressure or innovative extraction methods [60]. Advantages and drawbacks of traditional and innovative extraction techniques are summarized in Table 11.

Table 11. Advantages and drawbacks of traditional and innovative extraction techniques [60]

Extraction method	**Soxhlet**	**Supercritical extraction**	**Pressurized liquid extraction**	**Ultrasound assisted extraction**
Description	Sample is contained in an extraction cartridge and percolated with recondensed vapors of the solvent	Sample is placed in a high pressure vessel and crossed continuously by the supercritical fluid	Sample is heated by a conventional oven and crossed by the extraction solvent under pressure	Sample is immersed in solvent and submitted to ultrasound using a US probe or US bath
Extraction time	3–48 h	10–360 min	10–30 min	10–60 min
Sample size	1–30 g	1–5000 g	1–30 g	1–30 g
Solvent use	150–500 mL	2–5 mL (solid trap); 30–60 mL (liquid trap)	15–60 mL	50–200 mL
Investment	Low	High	High	Low
Advantages	Easy to handle, no filtration necessary, high matrix capacity	Fast extraction, low solvent consumption, concentration of the extract, no filtration necessary, possible high selectivity	Fast extraction, no filtration necessary, low solvent consumption	Easy to use
Drawbacks	Long extraction time, large solvent volume	Many parameters to optimize	Possible degradation of thermolabile analytes	Large solvent volume, filtration step required

To obtain bioactive compounds from plants one of traditional techniques is Soxhlet extraction (the temperature of the heating mantle must be some degrees above the boiling point of the solvent), when the liquid reaches the overflow level, a siphon aspirates the solute from the thimble-holder and unloads it back into the distillation flask, thus carrying the extracted analytes into the bulk liquid (Fig.8). This operation is repeated until extraction is complete. Soxhlet extraction was designed mainly for extraction of lipid and now it is used as a model for the comparison of new extraction alternatives [54, 61]. During Soxhlet extraction samples are usually extracted at the solvent boiling point over long periods, which can result in thermal

decomposition of thermolabile target species. Also, a conventional Soxhlet device provides no agitation, which would help to accelerate the process. In addition, after extraction, the second step, solvents evaporation is needed [54].

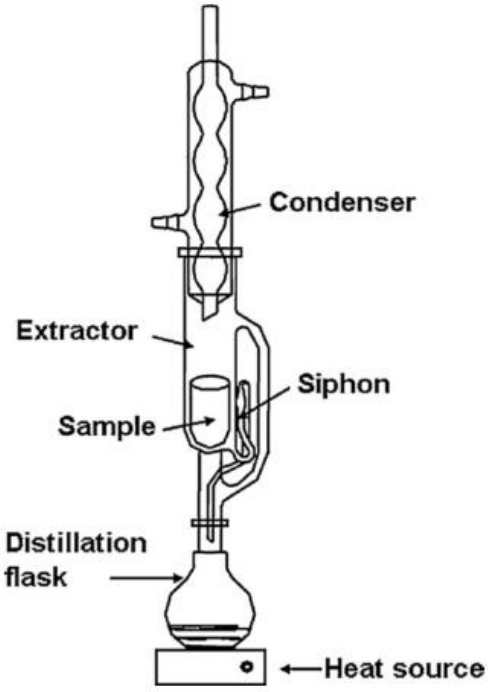


Figure 8. Conventional Soxhlet extractor [54]

When sample is solid and the required phase for analysis is a liquid, the process is called solid-liquid extraction (SLE). Solid-liquid extraction is one of the oldest known processes to isolate substances from plants. A simple and broadly applicable form of solid-liquid extraction entails combining the solid with a solvent in which the analyte is soluble. Through agitation, the analyte partitions into the liquid phase, which may then be separated from the solid through filtration. The choice of solvent must be made based on the solubility of the target analyte, and on the balance of cost, safety, and environmental concerns (Table 12) [45]. Table 12 presented that the major target compounds in elderberry were cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside, cyanidin 3-glucoside, quercetin, quercetin 3-*O*-rutinoside, quercetin-3-*O*-glucoside, 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, while total polyphenols were in range of 371–432 mg GAE/100 g FW, anthocyanins amounted in 273 mg/100 g FW; 664–1816 mg CGE/100 g FW or 8.33–101.40 mg CGE/g DW and cyanidin yielded in 3316 mg/kg FW.

Table 12. Isolation of phenolic compounds from *Sambucus nigra* L. berry fruits, using SLE [24]

Origin of berry fruits	Extraction conditions	Target compound	Yield	Ref.
Turkey	80 °C; 40 mL of 80% EtOH	Total polyphenols	371–432 mg GAE/100 g FW	[62]
		Anthocyanins total/sum	242–283 mg CGE/100 g FW	
Romania	acidified MeOH	Anthocyanins total/sum	273 mg/100 g FW	[63]
		Cyanidin 3-sambubioside-5-glucoside	23.34 mg/100 g FW	
		Cyanidin 3,5-diglucoside	47.1 mg/100 g FW	
		Cyanidin 3-sambubioside	134.94 mg/100 g FW	
		Cyanidin 3-glucoside	44.83 mg/100 g FW	
		Quercetin	0.55 mg/100 g FW	
		Quercetin 3-O-rutinoside	29.02 mg/100 g FW	
		Quercetin 3-O-glucoside	8.69 mg/100 g FW	
Denmark	acetonitrile and trifluoracetic acid 1.5 h	Anthocyanins total/sum	664–1816 mg CGE/100 g FW	[64]
		Cyanidin 3-sambubioside-5-glucoside	4–47 mg CGE/100 g FW	
		Cyanidin 3,5-diglucoside	5–36 mg CGE/100 g FW	
		Cyanidin 3-sambubioside	269–656 mg CGE/100 g FW	
		Cyanidin 3-glucoside	361–1266 mg CGE/100 g FW	
		Quercetin	29–60 mg/100 g FW	
Austria, Denmark	20 mL 50% MeCN; leaving sample at 22 °C for 2 h	Anthocyanins total/sum	8.33–101.40 mg CGE/g DW	[62]
		Cyanidin 3-sambubioside-5-glucoside	0.86–11.5 mg CSE/g DW	
		Cyanidin 3,5-diglucoside	0.12–5.22 mg CSE/g DW	
		Cyanidin 3-sambubioside	4.62–40.30 mg CSE/g DW	
		Cyanidin 3-glucoside	2.74–49.5 mg CSE/g DW	
		Quercetin 3-O-rutinoside	1.94–6.31 mg QRE/g DW	
		Quercetin 3-O-glucoside	0.11–1.08 mg QRE/g DW	
		3-O-Caffeoylquinic acid	0.05–0.40 mg ChAE/g DW	
		5-O-Caffeoylquinic acid	0.53–1.22 mg ChAE/g DW	
Finland	ethyl acetate	Cyanidin total/sum	3316 mg/kg FW	[65]
		Quercetin	331 mg/kg FW	

Conventional solid-liquid extraction (SLE) techniques and Soxhlet extraction, are time consuming and during them need to use large amounts of solvents, while major of food ingredients are known to be thermally sensitive. Several innovative extractions could replace conventional techniques and they ultrasound-assisted extraction (UAE), subcritical and supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and pressurized liquid extraction (PLE), or alternative pre-treatments, including high-pressure processing and pulsed electric field [60, 66].

1.5.2. SFE-CO_2 extraction

The supercritical extraction of raw materials is a large field of research, innovation and entrepreneurial developments [67]. The use of the supercritical SFE-CO_2 extraction process is a promising alternative that can achieve comparable oil yield with respect to the conventional organic solvent extraction with better product quality similar to that of mechanical pressing [68]. Fluid can become supercritical fluid, when it is forced to exact temperature and pressure above its critical point. There are a wide range of solvents that can be used as supercritical fluids, but

carbon dioxide is highly regarded because of its moderate temperature (30.9 °C) and pressure (7.37 MPa) (Fig.9) [69, 70].

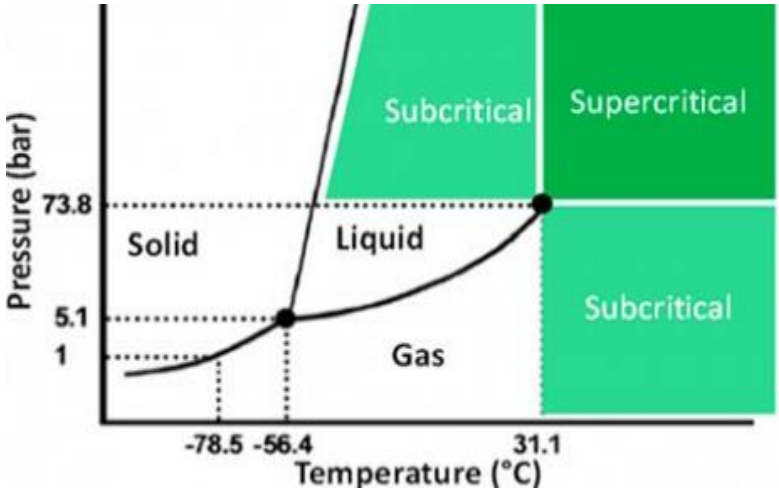


Figure 9. Pressure and temperature phase diagram for carbon dioxide [71]

The SFE apparatus (Fig.10) used for the extraction consists of an extractor filled with sample particles. CO_2 is delivered by a high-pressure pump. The instantaneous CO_2 flow rate and the total quantity of CO_2 used are usually measured by calibrated rotameters and test meters, respectively. When an extraction experiment starts, the extractor is pressurized with CO_2 first, then, when the operating pressure has been reached, CO_2 is allowed to flow continuously through the extractor at the set flow rate [72].

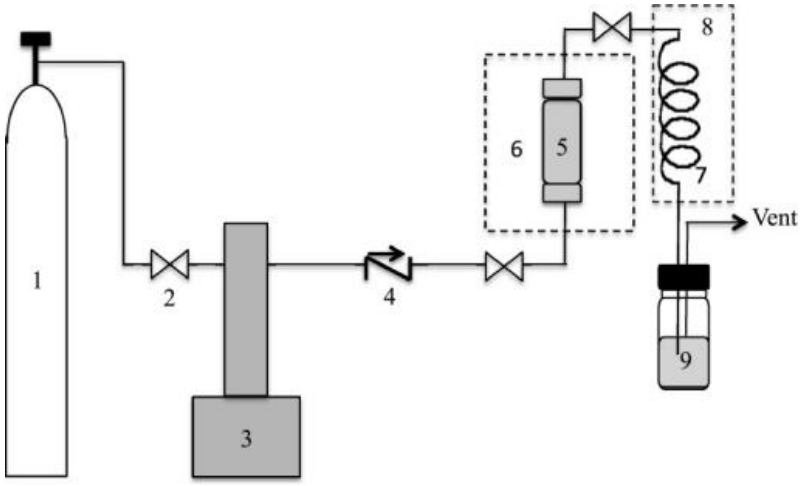


Figure 10. Scheme of the SFE-CO_2 equipment: (1) Liquid CO_2 cylinder with a dip tube; (2) valve; (3) CO2 syringe pump with a cooling jacket; (4) check valve; (5) extraction vessel; (6) extraction oven; (7) restrictor; (8) heater; and (9) collection vial with solvent [73]

SFE can be easily used in combination with other extraction techniques (for example, with PLE (Chapter 1.5.2.) to perform multi-step valorisation processes in which the raw material is previously extracted with CO_2 – to remove lipophilic compounds – and subsequently with water, alcohol or the mixture of both – to obtain a phenolic rich fraction [74].

The SFE-CO_2 (15 min static and 40 min dynamic extraction period; 20 MPa and 40 °C temperature) from the *S. nigra* pomace resulted in a lipophilic fraction with yield equal to 17.1%. The highest second step yield (24.2%) was obtained when no CO_2 was present in the extraction solvent (0:20:80). Furthermore, there were high PLE extraction yields obtained with high CO_2 amounts, in particular CO_2/EtOH/H_2O (90:2:8) and (80:1:19) (Table 13) [74].

Table 13. *Sambucus nigra* L. pomace extractions at 40 °C: experimental conditions, global yield, composition, and antioxidant activity of obtained extracts [74]

Extraction method	Operational conditions	Solvent mixture[a] (%)	Pomace humidity	Yield, %
SFE	55 min	CO_2 (100)	61%	2.8
SFE	55 min	CO_2 (100)	8.5%	17.2
		EtOH–H_2O (90) + CO_2 (10)		16.8
		EtOH (100)		12.7
		EtOH–H_2O (100)		18.2
SFE	55 min	CO_2 (100)	6.1%	17.4

[a]: EtOH–H_2O mixture had always a fixed proportion of 8:2 (v/v).

High antioxidant activity anthocyanin-rich extracts were successfully obtained from elderberry pomace using CO_2 and diverse CO_2/EtOH/H_2O mixtures in a fractionated high-pressure extraction methodology. The CO_2/EtOH/H_2O solvent composition had a great influence on extract yield and composition, in terms of total phenolic compounds, total flavonoids, anthocyanins and rutin [74]. However, to the best of our knowledge tour study, little information is so far available on elderberry pomace and in the study of Seabra et al. (2010), SFE-CO_2 conditions were not optimized for lipophilic fraction isolation from elderberry pomace [8].

1.5.2. Pressurized liquid extraction

The novel techniques offer the ability to reduce or to eliminate the use of toxic solvents, while improve process efficiency and enhance extraction yields. Innovative techniques can also be used as a pre-treatment or in combination with environment friendly, safe organic solvents to enhance extraction efficiency by improving cell-membrane permeability, which is the parameter governing extraction efficiency [60, 66]. Pressurized liquid extraction (PLE) is gaining importance and has been widely employed for the extraction of natural bioactive compounds from natural sources. PLE is a technique that involves extraction using liquid solvents at elevated temperature and pressure, which enhance the extraction performance as compared to those techniques carried out at near room temperature and atmospheric pressure. This technique is also known as pressurized liquid extraction; pressurized solvent extraction; accelerated solvent extraction and enhanced solvent extraction. There are two main set-ups for PLE, static and dynamic instruments. In the dynamic setup, the extraction solvent is continuously pumped

through the sample vessel. In the static set-up, the extraction process consists of one or several extraction cycles with replacement of the solvent between cycles (Fig. 11).

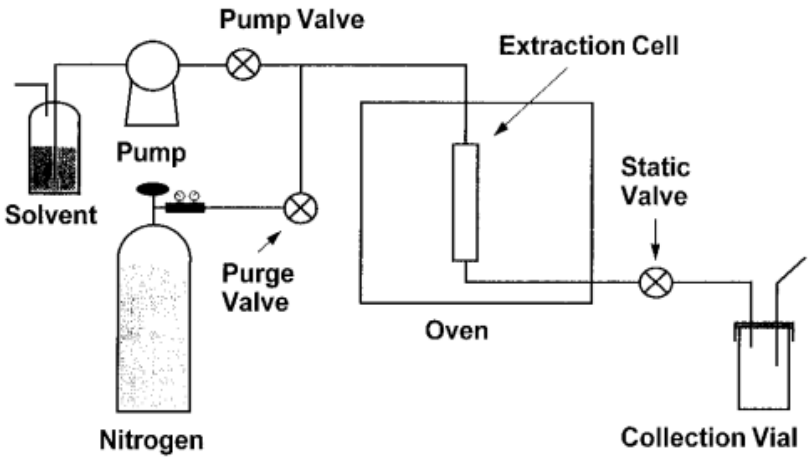


Figure 11. Schematic of pressurized liquid extraction system [75]

A wide range of extraction temperatures can be applied in PLE, it usually ranges from room temperature to 200 °C and the pressure range is 0.35–20 mPa. The efficiency of the extraction depends on the solubility of the analyte in the extraction solvent and on the partitioning of the target-compound between the water and the extraction solvent [76]. In comparison to the traditional Soxhlet extraction PLE was found to dramatically decrease time consumption and solvent use [61].

1.5.3. Ultrasound-assisted extraction method

Use of ultrasound (Fig.12) is a novel extraction technology for various molecules and biomaterials, including polysaccharides, essential oils, proteins, peptides and bioactive molecules of commercial importance. UAE can benefit the extraction process in many ways: enhancing extraction yield, rates with or without using solvents, providing the opportunity to use alternative (GRAS) solvents by improving their extraction performance and enhancing extraction of heat-sensitive components.

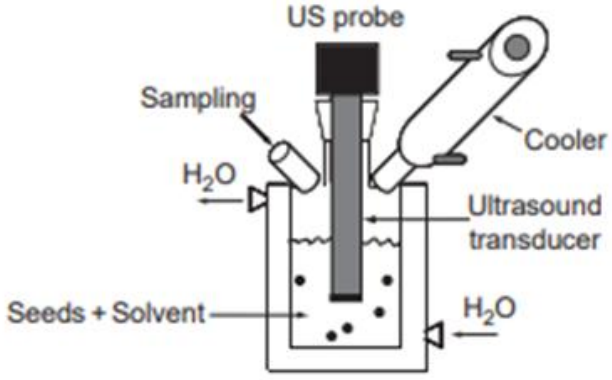


Figure 12. Scheme of ultrasound assisted extraction [60]

The main driving force for the extraction effects of sonication is acoustic cavitation. When ultrasound propagates through any medium, it induces a series of compressions and rarefactions in the molecules of the medium. Such alternating pressure changes cause the formation and, ultimately, the collapse of bubbles in a liquid medium. This phenomenon of creation, expansion, and implosive collapse of microbubbles in ultrasound-irradiated liquids is known as "acoustic cavitation". Of course, ultrasound-assisted extraction can be combined with other novel extraction technologies, such as supercritical fluid extraction, microwave and high-pressure extraction [66].

Table 14. Phenolic compounds yields, using UAE method, for target compounds isolation from *S. nigra* fruits [24]

Origin	Extraction method	Extraction conditions	Target compound	Yield	Ref.
USA	Ultrasonic vibration	10 min with acidified MeOH	Total polyphenols	364–582 mg GAE/100	[77]
			Anthocyanins total/sum	170–343 mg CGE/100 g	
			Cyanidin 3-sambubioside-5-glucoside	16–59 mg CGE/100 g FW	
			Cyanidin 3,5-diglucoside	8–20 mg CGE/100 g FW	
			Cyanidin 3-sambubioside	122–269 mg CGE/100 g FW	
			Cyanidin 3-glucoside	205–481 mg CGE/100 g FW	
			Flavonols total/sum	57.0–102.7 mg QRE/100 g	
			Quercetin 3-O-rutinoside	43–96 mg QRE/100 g	
			Quercetin 3-O-glucoside	4–15 mg QRE/100 g	
			Kaempferol 3-O-rutinoside	0.7–1.2 mg QRE/100 g	
			3-O-Caffeoylquinic acid	0.7–4.4 mg ChAE/100 g	
			4-O-Caffeoylquinic acid	1.2–2.5 mg ChAE/100 g	
			5-O-Caffeoylquinic acid	26.4–35.9 mg ChAE/100 g	
Slovenia	Ultrasonic vibration	with 20 ml MeOH containing 1% HCl and 1% BHT, 30 min	Anthocyanins total/sum	603–1265 mg CGE/100 g FW	[25]
			Cyanidin 3-sambubioside-5-glucoside	20–54 mg CGE/100 g FW	
			Cyanidin 3,5-diglucoside	7.4–23.3 mg CGE/100 g FW	
			Cyanidin 3-sambubioside	270.8–630.8 mg CGE/100 g FW	
			Cyanidin 3-glucoside	221.4–586.4 mg CGE/100 g FW	
			Cyanidin 3-rutinoside	1.5–9.6 mg CGE/100 g FW	
			Quercetin total/sum	73–52 mg/100 g FW	
			Quercetin	2.7–4.5 mg/100 g FW	
			Quercetin 3-O-rutinoside	3–52 mg/100 g FW	
			Quercetin 3-O-glucoside	6.–26.5 mg/100 g FW	
	Ultrasonic vibration	40 °C and 60 °C	Gallic acid	3.91 and 4.01 μg/g of DW	[42]
			Protocat.	63.82 and 65.25 μg/g of DW	
			4-OH-benzoic acid	6.03 and 6.54 μg/g of DW	
			Vanilic acid	0.92 and 1.01 μg/g of DW	
			Caffeic acid	2.23and 2.34 μg/g of DW	
			p-Coumaric acid	6.22and 6.40 μg/g of DW	
			Ferulic acid	1.19 and 1.28 μg/g of DW	

CGE, cyanidin 3-glucoside equivalents; ChAE, chlorogenic acid equivalents; CSE, cyanidin 3-sambubioside equivalents; DW, dry weight; FW, fresh weight

Table 14 presented that during ultrasound-assisted extraction could be extracted: total polyphenols 364–582 mg GAE/100 g, anthocyanins 170–343 mg CGE/100 g and 603–1265 mg CGE/100 g FW; while the major phenolic acids in elderberry were: gallic acid (3.91 and 4.01 μg/g of DW), 4-OH-benzoic (6.0 and 6.5 μg/g of DW), vanilic (0.9 and 1.0 μg/g of DW), caffeic (2.2 and 2.3 μg/g of DW), p-Coumaric (6.2 and 6.0 μg/g of DW) and ferulic acids (1.2 and 1.3 μg/g of DW). Recently, Oniszczuk et al. (2016) asserted that *S. nigra* fruits can be potential component of functional food and made the snacks enriched with *S. nigra* fruits. Detailed qualitative and quantitative analysis, using PLE and UAE extraction methods, was performed of phenolic acids composition. It was found that very important step of the analysis is selection and optimization of the extraction method. In this case, the most effective extraction method of phenolic acids from snacks was UAE at 60°C. UAE method offered high yield of analysed compounds in short times and reduced volume of solvent [42].

1.5.4. Future trends and perspectives

Elderberry juice processing creates about 25-40% of residues (peels, seeds), that are used as fertilizer, animal feed or just thrown away. However, pomace after juice processing is still valuable source of bioactive compounds and polyunsaturated fatty acids [8], that have positive effect on human health. Non-polar and polar fractions from elderberry pomace could be isolate during conventional (Soxhlet, SLE) and alternative extractions (SFE-CO_2, PLE, UAE), but after the literature data tour, it was noticed, that researches, which are related with elderberry pomace, are just a few. Further, the attention to wastage after fruits processing are increasing, but still is needed to investigate the optimal non-conventional extraction parameters for biorefining of elderberry pomace.

2. MATERIALS AND METHODS

2.1. *Sambucus nigra* L. berries and pomace

Sambucus nigra L. berries was obtained from the JSC "Fudo" (Kaunas, Lietuva). Elderberries were frozen (-18 °C) by the manufacturer and transferred in the cooler bags. Berries were unfreezed and squeezed juice. Pomace was dried up at 40 ºC temperature and ground by an ultra centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.5 mm hole size sieve. Juice was freeze-dried and both, juice and pomace, was kept in tightly closed, dry glass jars, in dark, well-ventilated place prior to the analysis.

2.2. Chemicals

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$, Sigma-Aldrich, Steinheim, Germany), 3,4,5-trihydroxybenzoic acid (gallic acid, 99%, Sigma-Aldrich, Steinheim, Germany), 2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoic acid (Fluorescein (FL), Fluka Analytical, Bornem, Belgium), 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%, Sigma-Aldrich, Steinheim, Germany), Folin & Ciocalteu's phenol reagent ((2M), Fluka Analytical, Bornem, Belgium), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (Lach-Ner, Brno, Czech Republic), Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), Na_2CO_3 (Sigma-Aldrich), H_2SO_4, NaOH, acetonitrile, methanol, hexane (HPLC grade, Sigma-Aldrich Chemie, Steinheim, Germany), boron trifluoride (24% methanol solution, Acros organics, Geel, Belgium), L-prunasin standart, a mixture of C_7-C_{30}-Saturated Alkane Std (Sigma Chemical Co., St. Louis, MO), microcrystalline cellulose (20 μm, Sigma-Aldrich, St. Louis, MO, USA), catalytic tablet (K_2SO_4, $CuSO_4$, Sigma-Aldrich), ASE filters (Glass Fiber_(X)_Cellulose, Dionex Corporation, Sunnyvale, CA,USA), diatomaceous earth (100 % SiO_2, Dionex Corporation, Sunnyvale, CA, USA), cotton-wool (Bella-cotton, Poland), ethanol (96.3%, food grade, Stumbras, Kaunas, Lithuania), nitrogen liquid (AGA SIA, Riga, Latvia), carbon dioxide gases and nitrogen gases (99.9%, Gaschema, Jonava region, Lithuania). All solvents were of analytical and HPLC-grade grade.

2.3. Determination of the selected chemical composition indices

2.3.1. Moisture content

To the heated, dry, constant weight glass with cap and rod, 4.87 ± 0.002 g of *S. nigra* pomace (particle size 0.5 mm) and 4.640 ± 0.002 g of berries (particle size 0.5 mm) were added and dried in the oven at 100-105°C for 3 hours, afterwards placed in a desiccator for 25 minutes

and weighted on the analytical balances. The heating-weighting procedure afterwards was repeated every hour until variation between two weighting results was less than 0.005 g. Experiments were performed in duplicate. Moisture content was calculated using the formula below (g/100g).

$$x = \frac{(m_1 - m_2)*100}{m_1 - m}; g/100g \qquad (1)$$

m – glass with cap and rod weight g; m_1 – glass weight with sample before drying g; m_2 – glass weight with sample after drying, g.

2.3.2. Mineral content

2.700 ± 0.002 g of *S. nigra* pomace (0.5 mm fraction) and 3.020 ± 0.002 g of berries (0.5 mm fraction) was added to dry, constant weight crucible, heated on electric hotplate for 20 minutes and kept in muffle for ~16 hours at 600-650 °C, afterwards placed in a desiccator for 25 minutes and weighted on the analytical balances. The heating-weighting procedure was repeated until variation between two weighting results was less than 0.005 g. Experiments were performed in duplicate. Ash (mineral) content, expressed as a percentage, is calculated by the following formula:

$$x = \frac{(m_2 - m)*100}{m_1 - m}; g/100g \qquad (2)$$

m – crucible weight, g; m_1 – crucible weight with sample, g; m_2 – crucible weight with burned sample, g.

2.3.3. Protein content by Kjeldahl method

To a Kjeldahl flask, 1.000 ± 0.002 g of pomace and berries (0.5 mm fraction), 20 ml of 98% conc. H_2SO_4 and catalyst tablet, containing 3.5 g K_2SO_4 and 0.4 g $CuSO_4$, were added, and mineralized until solution in the flask became transparent (heating intensity 60%, time – 90 min). The solution was distillated with automatic steam distillation system under the following conditions: 3 s NaOH and 3 s H_3BO_4 filing parameters, distillation time 300 min, steam intensity 80%. Distillate was collected in flask, followed with the addition of Tashiro indicator and titration with 0.01 N HCl until the colour change from light green to grey-violet. Experiments were performed in duplicate. Control sample, only distilled water, was prepared and analysed following the above described conditions. The nitrogen content, expressed as a percentage, was calculated using the following formula:

$$x = \frac{0.0014*A}{m} * 100; g/100g \qquad (3)$$

A – 0.1N HCl amount, used for distillate titration, ml; m – sample weight, g; 0.0014 – nitrogen amount equivalent 1 ml 0.1 N HCl. Protein material amount is calculated by multiplying the amount of nitrogen from the conversion factor 6.25.

2.4. Conventional extraction techniques

2.4.1. Soxhlet extraction

Soxhlet extraction was performed from 18.060 ± 0.001 g of ground pomace (0.5 mm) and from 4.050 ± 0.001 g ground berries (0.5 mm) inserted into an inner tube (rolled up tightly in filter paper) of the in an automated Soxhlet extractor EZ100H apparatus (Behr Labor-Technik, Düsseldorf, Germany). Non-polar fractions from pomaces and berries were isolated using hexane. Pomaces (0.5 mm fraction) were further extracted with acetone and then with ethanol. The rate of extraction was 1 cycle per 5 min, total extraction time – 360 min (6 hours) for each step extraction. Organic solvents were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland). Extract yields (Soxhlet-He; Soxhlet-He-Ac; Soxhlet-He-Ac-EtOH) were determined gravimetrically (±0.001 g) and expressed as g/100 g pomace. Dry extracts were kept under the nitrogen flow for 15 min to remove organic solvent residues and stored at -18°C prior to the analysis. The solid residue was collected, dried (50 °C) in and kept in a dry, well-ventilated place prior to the analysis. Experiments were performed in duplicate.

2.4.2. Solid-liquid extraction (SLE)

SLE was performed in a thermostatically controlled shaker from 15.000 ±0.100 g of pomace (0.5 mm fraction) and 150 mL of hexane, acetone, ethanol and distilled water (*solid: liquid* ratio *1:10*) at the following conditions: temperature 60 °C (for hexane extraction: SLE-He), 40 °C (for acetone extraction: SLE-He-Ac), 60 °C (for ethanol and aqueous extraction: SLE-He-Ac-EtOH, SLE-He-Ac-EtOH-H_2O, respectively), time 360 min, 800 rpm, followed by the rapid cooling and centrifugation (9000 rpm, 10 min) and filtration. Organic solvents from the optically clear supernatants were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland) and aqueous supernatants were freeze-dried. Dry extracts were kept under the nitrogen flow for 15 min to remove organic solvent residues and stored at -18°C prior to the analysis. SLE-He, SLE-Ac, SLE-EtOH and SLE-H_2O extracts yield was determined gravimetrically (±0.001 g) and expressed as g/100 g pomace. The solid residues were collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis.

2.5. High-pressure extraction techniques

2.5.1. Supercritical CO_2 extraction (SFE-CO_2)

SFE-CO_2 was performed in a supercritical fluid extractor Helix extraction system (Applied Separation, Allentown, PA, USA) by modified procedure of Kraujalis and Venskutonis (2013) [78]. Each extraction was carried out using 5.000 ± 0.002 g of ground pomace material (0.5 mm), which was placed in a 50 mL cylindrical extractor (14 mm inner diameter and 320 mm

length) in between two layers of the cotton wool to avoid particle release to the system. Cylindrical extractor temperature was controlled by the surrounding heating cover. The volume of CO_2 consumed was measured by a ball float rotameter and a digital mass flow meter in standard litres per minute (SL/min at standard state (P_{CO2} = 100 kPa, T_{CO2} = 20°C, ρ_{CO2} = 0.0018 g/ml). The flow rate of CO_2 was controlled manually by the micro-metering valve and kept at 1.8-2.2 SL/min during all experiments. The static extraction time was 10 min for all experiments.

Response surface methodology (RSM) and central composite design (CCD) were utilized for the experimental design setup of SFE-CO_2. Three independent variables and their variation levels were chosen, as follows: pressure (15-50 mPa), temperature (40-70 °C) and dynamic extraction time (30-120 minutes).

Table 15. Levels of independent variables for SFE-CO_2 parameter optimization

Experimental factors	**Variable levels**		
	-1	*0*	*+1*
Extraction temperature (T, ºC)	40	55	70
Extraction time (τ, min)	30	75	120
Extraction pressure (P, mPa)	15	35	50

The response factor (RF) was the total yield of SFE-CO_2 extract. The number of experiments was defined, based on the equation 1:

$$N = 2^f + 2f + c \qquad (1)$$

Where: f - the number of factors; c – the number of centre points.

Complete design consisted of 20 experimental runs with 8 factorial points, 6 axial and 6 centre points was established using the software Design-Expert trial version 8.0.7.1 (Stat–Ease Inc., Minneapolis, MN). The multiple regression equation was used to fit the second-order polynomial equation, expressing the yield of SFE-CO_2 extract as a function of independent variables (Equation 2):

$$Y = \beta 0 + \sum_{i=1}^{3} \beta iXi + \sum_{i=1}^{3} \beta iiXi^2 + \sum_{i=1}^{3} \sum_{j>1}^{3} \beta ijXiXj \qquad (2)$$

Where: Y – the predicted response (SFE-CO_2 extract yield, g/100 g DW); β_0 – a constant; β_i, β_{ii} and β_{ij} – the coefficients for linearity; X_i and X_j – independent variables.

After extractions, the yield of extracts was determined gravimetrically (±0.001 g) and expressed as g/100g pomace. Extracts were collected to an opaque bottle and kept in a freezer until further analysis. Solid residues were kept dry, well-ventilated place prior to the further analysis.

All extraction experiments were performed in duplicate and in random order. Results were analysed using Design-Expert trial version 8.0.7.1 software. Statistical significance of the model

and each variable was determined using the Student test (*p*-value) at 5% probability level ($p < 0.05$). The adequacy of the model was determined by evaluating the '*lack of fit*' coefficient and the Fisher test value (*F*-value) obtained from analysis of variance. SFE-CO_2 kinetics was performed at 55 °C, 35 MPa and the extraction yields were analysed every 15 min till 120 min, determined gravimetrically (±0.001 g) and expressed as g/100g DW. All extractions were performed in duplicates.

2.5.2. Pressurised liquid extraction (PLE)

PLE was performed in ASE-350 (Thermo Scientific Dionex, Sunnyvale, CA, USA) apparatus following modified procedure of Oniszczuk et al. (2016) [42] from 3.000 ±0.001 g of ground pomace or residue after SFE-CO_2 (0.5 mm) was mixed with 1.000 g diatomaceous earth (3/1, w/w) and placed to 66 ml stainless-steel extraction cells, with two cellulose filters in the both ends to avoid particle release to the system. In order to compare conventional and high-pressure extraction efficiencies, elderberry pomace was extracted with: a) hexane (extraction temperature 60 °C, time 5min x 3 cycles and 10 min x 3 cycles); b) the residue after SFE-CO_2 was extracted with: ethanol, under the following extraction conditions: temperature 60 °C, time 5 min x 3 cycles and 10 min x 3 cycles and c) residues after PLE-EtOH were further extracted with H_2O (extraction temperature 140 °C, time 5min x 3 cycles); d) the residue after SFE-CO_2 was extracted only with distilled water under the following conditions: extraction temperature 140 °C, time 5 min x 3 cycles and 10 min x 3 cycles.

The system pressure (103 bar or 10.3 MPa), pre-heating time (5 min), cell flush volume (100%) and purge time (120 s) with nitrogen to collect the extracts in the vials was kept constant for all PLE experiments. Organic solvents were evaporated with rotary evaporator at different pressure (hexane – 180 bar, ethanol – 80 bar) at 45 °C by Buchi Rotavapor R-210 (BUCHI Labotechnic, Switzerland). H_2O extracts were additionally freeze-dried (-50 ºC, 0.5 mbar) to remove residual water. The yields of extracts were determined gravimetrically (±0.001 g) and expressed as g/100g DW, extract was kept in brown glass bottles in the freezer prior to the analysis. The solid residues were collected, dried (50 °C) in and kept in a dry, well-ventilated place prior to the analysis. All extractions were performed in duplicates.

2.5.3. Ultrasound-assisted extraction (UAE)

UAE was performed with Ultrasonic Processor UP200Ht (Hielscher Ultrasonics, Teltow, Germany) apparatus following modified procedure of Oniszczuk et al. (2016) [42] from 4.000 ± 0.001 g of ground pomace (particle size 0.5 mm) and from 4.000 ± 0.001 g residue after SFE-CO_2 (particle size 0.5 mm). In order to compare conventional and innovative extraction efficiencies, *S. nigra* pomace was extracted with 40 ml hexane, residue of SFE-CO_2 was

extracted with 40 ml of ethanol, while residues after UAE-EtOH were further extracted with distilled water. The influence of time was investigated during extractions and after 5, 10, 15 and 20 min extracts were centrifuged (4500 rpm, 10 min) and the optically clear supernatant were evaporated with rotary evaporator at different pressure (hexane – 180 bar, ethanol – 80 bar) at 45 °C by Buchi Rotavapor R-210 (BUCHI Labotechnic, Switzerland). H_2O extracts were additionally freeze-dried (-50 ºC, 0.5 mbar) to remove residual water. The yields of extracts were determined gravimetrically (±0.001 g) and expressed as g/100g pomace, extracts were kept in brown glass bottles in the freezer prior to the analysis. The solid residues were collected, dried (50 °C) in and kept in a dry, well-ventilated place prior to the analysis. All extractions were performed in duplicates.

2.6. Antioxidant activity assessment of extracts and solid residues

For the *in vitro* antioxidant activity measurements in Folin-Ciocalteu's, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC assays, various extracts after different steps of extraction were dissolved in acetone-methanol mixture (1:9, v/v) and further diluted with methanol to a final concentration from 0.1 mg/mL to 50 mg/mL. Water-soluble fractions were dissolved in dist. H_2O to a final concentration from 10 mg/mL to 10 mg/mL.

Antioxidant capacity of starting plant material and solid residues after various steps of extraction was evaluated by QUENCHER method (Gökmen et al., 2009 [79]). As previously described by Kitrytė et al. (2015) [80], stock mixtures were prepared with 0.2 mm fractions and microcrystalline cellulose at a concentration of 0.500 mg/mg. Final solid dilution for analysis were prepared at concentrations of 0.002 mg/mg to 0.500 mg/mg.

2.6.1. Total phenolic content (TPC) by Folin-Ciocalteu's assay

Folin-Ciocalteu's assay was carried out by the procedure of Singleton, Orthofer and Lamueal-Raventós (1999) [81], with some modifications. For the analysis, 150 µL of sample (100-700 µg/mL) or MeOH (blank) were mixed with 750 µL Folin-Ciocalteu's reagent (2M), previously diluted with distilled water (1:9, v/v), and after 3 min of reaction, 600 µL of Na_2CO_3 solution (75g/L), left in dark for 2 hours and absorbance was measured at 760 nm with with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY).

For the QUENCHER procedure, as previously described by Kitrytė et al. (2014) [80], 10 mg of sample (0.050-0.005 mg/mg) or cellulose (blank) were mixed with 150 µL of distilled water, 750 µL Folin-Ciocalteu's, 600 µL of Na_2CO_3 solution, vortexed for 15 s, shaken at 250 rpm in the dark for 2 hours, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 760 nm with spectrometer. Gallic acid solutions (150 µL) at various concentrations (0-80 µg/mL) were used for calibration. The TPC of extracts and solid samples

was expressed as gallic acid equivalents (mg GAE/g sample and mg GAE/ g pomace) by means of dose-response curves for gallic acid. Extracts calibration curve:

$y = 0.0104x + 0.0185, R^2 = 0.9987,$

QUENCHER: $y = 0.0143x + 0.0098, R^2 = 0.99732.$

2.6.2. The ABTS$^{\bullet+}$ scavenging assay

The ABTS$^{\bullet+}$ assay was carried out by the method of Re at al. (1999) with slight modifications. Firstly, phosphate buffered saline (PBS) solution (75 mmol/L; pH 7.4) was preperad by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of distilled water. The ABTS$^{\bullet+}$ solution was prepared by mixing 50 mL of ABTS$^{\bullet+}$ (2 mmol/L PBS) with 200 µL $K_2S_2O_8$ (70 mmol/L) and allowing the mixture to stand in the dark at room temperature for 15-16 h before use. The working solution was prepared by diluting the ABTS$^{\bullet+}$ solution with PBS to obtain the absorbance of AU 0.700±0.010 at 734 nm. To a 1500 µL of working ABTS$^{\bullet+}$ radical solution 25 µL of sample (10-25 mg/mg) or MeOH (blank) were added, mixtures left in dark for 2 hours and absorbance was measured at 734 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY).

For the QUENCHER procedure, 10 mg of sample (0.05-0.005 mg/mg) or cellulose (blank) were mixed with 25 µL of MeOH and 1500 µL of working ABTS$^{\bullet+}$ radical solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 734 nm. Trolox solutions (25 µL) at various concentrations (0-1500 µmol/L MeOH) were used for calibration. $TEAC_{ABTS}$ of extracts and solid samples were calculated by means of dose-response curves for Trolox. Extract: $y = 0.0638x + 1.3042, R^2 = 0.9977$, QUENCHER: $y = 0.0625x + 2.804, R^2 = 0.9971$

2.6.3. Oxygen radical absorbance capacity (ORAC) assay

ORAC of the samples was evaluated as described by Prior et al. (2005) [82] by using fluorescein as a fluorescent probe. In the 96-well black opaque microplates, 25 µL sample or MeOH (blank) was mixed with 150 µL of fluorescein solution (14 µmol/L) preincubated for 15 min at 37°C, followed by a rapid addition of 25 µL of AAPH solution (240 mmol/L). The microplate was immediately placed in the FLUOstar Omega reader (BMG Labtech, Offenburg, Germany), automatically shaken prior to each reading and the fluorescence was recorded every cycle (1min x 1.1), total 120 cycles. The 485-P excitation and 520-P emission filters were used. Raw data were exported from the Mars software to Excel 2003 (Microsoft, Roselle, IL) for further calculations. Antioxidant curves (fluorescence versus time) were first normalized and from the normalized curves, the area under the fluorescence decay curve (AUC) was calculated

as $AUC = 1 + \sum_{i=1}^{i=150} \frac{f_i}{f_0}$, where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. $TEAC_{orac}$ of extracts were calculated by means of dose-response curve for Trolox. Extract: $y = 0{,}1394x - 0{,}7395, R^2 = 0.9977$.

2.6.4. Measurement of oxidation induction period by Oxipres

The effect of *S. nigra* pomace extracts on the oxidative stability of commercial rapeseed oil was tested by instrumental Oxipres method Trojáková et al., 1999 [83]; Basegmez et al. (2017) [4]. The samples were prepared by mixing rapeseed oil with SFE-CO_2 (0.5%, 1%), after Soxhlet with hexane extracts (1% and 5%). As control sample was used pure rapeseed oil. 5.000 ± 0.001 g of prepared (or control) sample was placed in a reactor tube and thermostated at 110°C under oxygen atmosphere at 5 bar in Oxipres apparatus (Mikrolab, Aarhus, Denmark), which measures pressure changes due to the absorption of oxygen consumed for oil oxidation. The induction period (IP) was calculated as the time after which the pressure began to decrease abruptly (its end was measured from the cross-section point of tangents of the first part and the subsequent part of the curve recording the pressure changes) [83]. Each measurement was done in duplicate.

2.7. Phytohemical characterization of extracts

2.7.1. Fatty acid composition analysis by gas chromatography (GC-FID)

Fatty acid composition analysis was performed by the procedure of Milinsk et al. [84] with some modifications. For triglycerides esterification and free acids saponification, 0.250 g extract (SFE-CO_2, Soxhlet-He, SLE-He, PLE-He-1, PLE-He-2, UAE-He) and 2 ml of methanolic NaOH (0.5 N) was poured into 50 ml round-bottomed flask and heated with condenser until disappearance of the fatty phase (5-10 min). After esterification, over the top of condenser 2.5 ml of 24% boron trifluoride/methanol complex was poured and boiled for 2 min., then cooled to room temperature. The sample was diluted with 2.5 ml n-hexane and the same amount of NaCl was added, well-shaken and left still until layers separated. The top hexane phase was collected with a Pasteur pipette and stored at 4ºC until analysis. For analysis, 100 µl of hexane phase was diluted with 900 µl pure GC-grade hexane. Analysis was carried out with gas chromatograph HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) using a flame ionization detector with a pole SPTM-2560 column (100 m long, 0,25 mm internal diameter the adsorbent layer of 0,20 µm (Supelco, Bellefonte, PA, USA). Oven temperature was programmed from 80°C to 240°C and increasing every 4°C/min. Injector temperature – 220°C and detector – 240°C. Injected amount of sample – 1µl. For compounds identification, a mixture of 37 fatty acids (SupelcoTM) were used as standards. Fatty acid methyl esters were identified by the retention time and the

percentage of fatty acid composition was calculated comparing peak areas to the corresponding reference compounds. All experiments were performed in duplicate.

2.7.2. Volatile compounds analysis by solid phase microextraction-gas chromatography-mass spectroscopy

S. nigra SFE-CO_2 extract, which was obtained at optimal conditions (35 MPa, 53 °C, 105 min) volatile compounds were extracted by solid phase microextraction (SPME), identified by gas chromatography-mass spectrometry (GC-MS). For analysis 4.000±0.001 g of ground berries, pomace and residue after SFE-CO_2 at optimal conditions were placed into vial for SPME extraction and experiments were performed in triplicate. The SPME conditions were: SPME fiber CAR™/ PDMS 85 µm (Supelco). Sample volume 1 ml, extraction temperature 35 °C, equilibrium time 30 min., extraction time 20 min., desorption temperature 250 °C, desorption time 5 min. Gas chromatograph used was a Perkin Elmer Clarus500 GC coupled to a Perkin Elmer Clarus 500 series mass selective detector (Perkin Elmer Instruments, Shelton, USA).

Volatile compounds were analyzed by GC-MS analysis, as previously described by Baranauskienė and Venskutonis (2013) [85]. GC–MS analyses were performed using a Perkin Elmer Clarus500 GC coupled to a Perkin Elmer Clarus 500 series mass selective detector (Perkin Elmer Instruments, Shelton, USA) in the electron impact ionization mode at 70 eV, the mass range was m/z 29–550. Volatile compounds were separated using an Elite-5 MS capillary column (dimethylpolysiloxane, 5% diphenyl), 30 m length, 0.25 mmi.d., 0.25 µm film thickness (Perkin Elmer Instruments, Shelton,USA). The oven temperature was programmed from 50 °C (2 min) to 250 °C (10 min) at the rate of 5 °C min^{-1}. Carrier gas helium was adjusted to a linear velocity of 36.2 cm s^{-1} at 50°C or 1.0 mL min^{-1} volumetric flow. Split mode was used at ratio of 1:20 and an injector temperature of 250 °C. The identity of the components was assigned by comparing their Linear retention indices (LRI) relative to C_8–C_{32n}-alkanes (SigmaChemical Co., St. Louis, MO), obtained on non-polar DB-5 column with those provided in literature (Adams, 2009) [86]. All experiments were done in duplicate.

2.7.3. Phytochemical characterization by UPLC/ESI-QTOF-MS

Phytochemical composition of berries, freeze-dried juice, pomace, Soxhlet extracts, SFE-CO_2 extract at optimal conditions, SLE extracts (SLE-He; SLE-He-Ac; SLE-He-Ac-EtOH), PLE extracts (PLE-He; PLE-EtOH; PLE-EtOH–H_2O), UAE extracts (UAE-He; UAE-EtOH) were screened on an Acquity UPLC system (Waters, Milford, USA) equipped with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germant), binary solvent delivery system, an autosampler with a 10 µL sample loop, column manager, photodiode array (PDA) detector and an Acquity BEH C18 column (1.7 m. 50 x 2.1 mm, i.d.), as previously described by

Kraujalyte et al. (2013) [87] with following modifications. The mobile phase initially consisted of eluent A (0.4 v/v formic acid in ultra-pure water), followed by an increase from 0% to 100% of eluent B (acetonitrile) over 9 min. During the following 2 min, the amount of eluent B was maintained at 100 %, then in 1 min, column was equilibrated initial conditions, were re-introduced for 1 min. Separation of compounds was performed at 25°C; the column was equilibrated for 2 min before each run; the flow rate was 0.4 mL/min; extract concentrations 1 mg/ml; injection volume 1 µL. The effluent (monitored at 254 nm) from the PDA detector was introduced directly into the UHR-TOF mass spectrometer equipped with an ESI source. MS data was recorded in two runs in ESI negative and positive ionization mode. The capillary voltage was maintained at +4000 V with the end plate offset at -500 V. Nitrogen was used as the drying and nebulizing gas at a flow rate of 10.0 L/min and a pressure of 2.0 bar. For the instrument control and data acquisition, the Compass 1.3 (HYStar 3.2 SR2) software was used. Preliminary peak identification was carried out by comparing accurate masses of compounds with hose reported in literature sources and free chemical databases (Metlin, Chempspider).

2.7.4. Quantitative analysis of sambunigrin

For the quantitative analysis of sambunigrin *S. nigra* freeze-dried juice, Soxhlet extracts, SFE-CO_2 extract at optimal conditions, SLE extracts (mechanical shaking with He–Ac–EtOH–dist. H_2O), PLE extracts (PLE-He; EtOH; EtOH–dist.H_2O and PLE-H_2O), UAE extracts (UAE-He; EtOH and EtOH-H_2O) were dissolved in methanol-ultrapure H_2O mixture (7:3, v/v) and further diluted with mixture to a final concentration from 10 mg/mL to 1 mg/mL. Solid ground *S. nigra* berries and pomace (0.5 mm), were performed by the procedure of Senica et al. (2016) [35] with some modifications. Solid sample was extracted with 5 mL of 700 mL/L MeOH with 300 mL/L ultrapure H_2O for 30 min at 30 °C in shaker. The samples were centrifuged for 7 min at 4 °C and 4800 rpm and the supernatants were filtered through a polyamide filter into vials prior to MS analysis. Analysis was performed on a Water Xeco TQ-S triple quadrupole mass spectrometer. Compounds were introduced to the spectrometer, using Waters H-class UPLC system, consisting of quaternary solvent pump, auto sampler and column thermostat. Chromatographic elution gradient and column were used as described earlier. Daughter spectra of every eluted compound were recorded in ESI negative ionization daughter scan mode for *m/z* values of corresponding compounds: parent *m/z* was 318.1305 and daughter *m/z* was 256.1224 and quantification trace – 318.131 > 256.122; predicted retention time – 5.15 ± 0.15 s; flow rate – 0.4 mL/min; column temperature – 40 °C; solvents: (A) 0.4% formic acid; (B) acetonitrile (Table 16):

Table 16. Acenotitrile concentration depending on time in sambunigrin analysis

Time, min	% B
0	0
8	100
9	100
9.2	0
11	0

Dwell was – 0.025 s; cone voltage – 56 V; collision energy – 22 V, collision gas flow 0.15 mL/min; desolvation gass flow – 1000 L/h, cone gas flow – 150 L/h; mebulizer pressure – 7.0 bar.2.8.

The calibration curve for sambunigrin quantification was:

$252551 * \times +5759.93; r = 0.999979$

2.7.5. Statistical analysis

Mean values and standard deviations were calculated using MS Excel 2016. One-way analysis of the variance (ANOVA), followed by the Tukey's posthoc test to compare the means that showed significant variation ($p < 0.05$ using GraphPad Prism 7 software (2012).

3. RESULTS AND DISCUSSION

Based on the reported literature data (Chapter 1) mostly *S. nigra* berries and juice are under study. However, little information is so far available on elderberry pomace [88]. Though, several authors predicated that *S. nigra* pomace could be a potential cheap and alternative source of natural bioactive non-polar and polar constituents for other, food, pharmaceutical or cosmetic industries [13].

In this work, we have also attempted to establish to find evidence, which supporting the need to use *S. nigra* pomace for isolation valuable non-polar and polar constituents. For this reason, conventional, namely Soxhlet, solid-liquid extraction (SLE), and innovative, such as supercritical fluid extraction with CO_2 (SFE-CO_2), pressurized-liquid extraction (PLE) and ultrasound-assisted extraction (UAE), were used for target compounds isolation.

Non-polar compounds were isolated with hexane, excepting SFE-CO_2, where was used only CO_2, and polar compounds were isolated with acetone, ethanol and water. Innovative extraction methods were compared with conventional extraction methods, in order to find is it possible to shorten the extraction time, reduced solvents use, and increased target compounds yields.

After all extractions, target solid residues and extracts were explored and determined: moisture, dry matter, lipid, protein contents of *S. nigra* berries and pomace; optimal SFE-CO_2 parameters for isolation non-polar fraction from elderberry pomace after juice processing and was developed the best multi-step scheme for both, non-polar and polar, fractions isolation, also was determined fractions yields after conventional, high-pressure and ultrasound-assisted extraction; fatty acid composition of non-polar extracts by GS-FID, *in vitro* antioxidant capacity (TPC, $ABTS^{\bullet+}$, ORAC, oxipres); volatile compounds profile of SFE-CO_2 extract at optimal conditions (35 MPa, 53 °C; 105 min) by GS-MS and preliminary phytochemical composition by UPLC-QTOF-MS were determined for target non-polar and polar extracts (Fig.13).

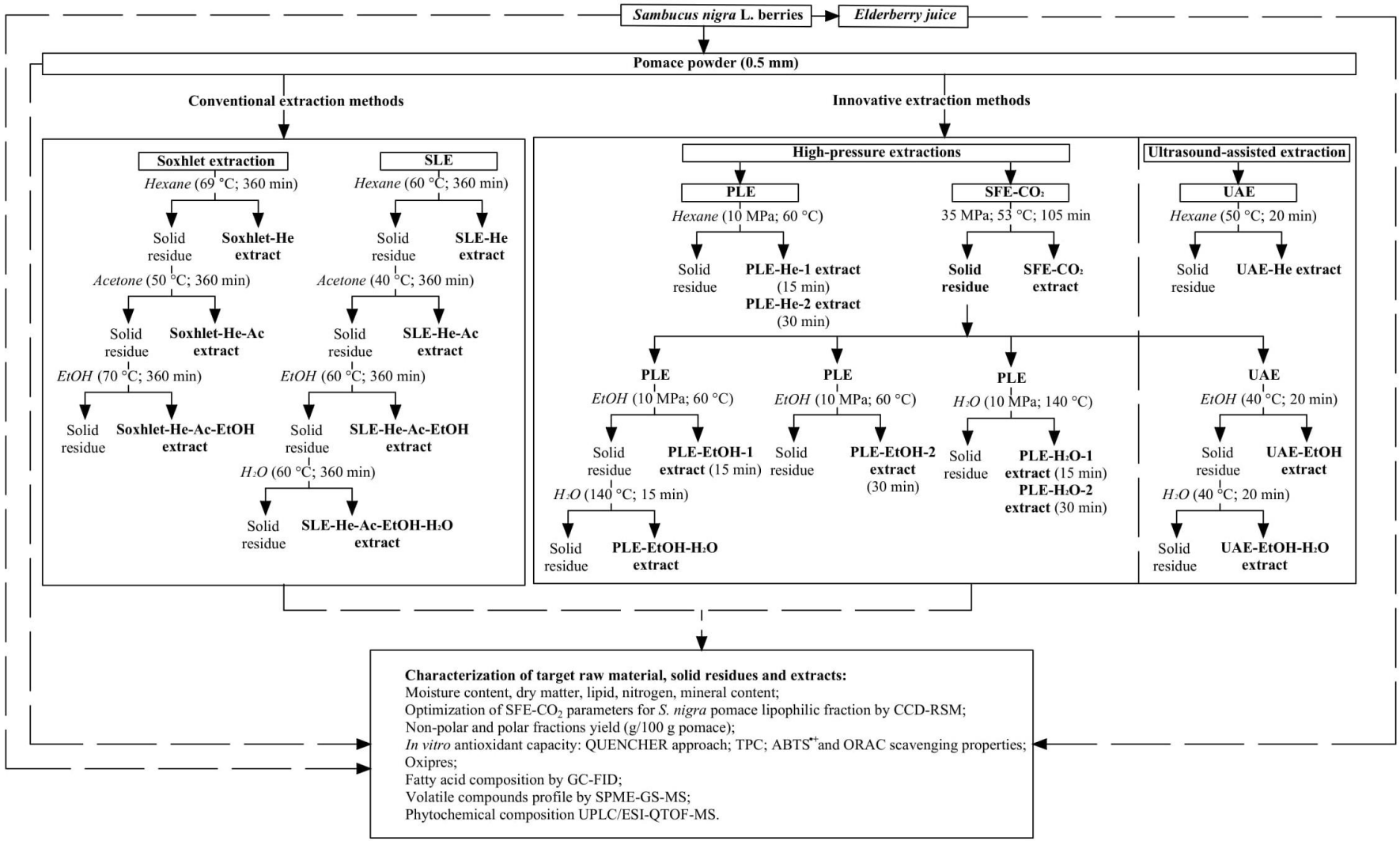


Figure 13. Scheme of analysis of *Sambucus nigra* L. berries, juice and pomace

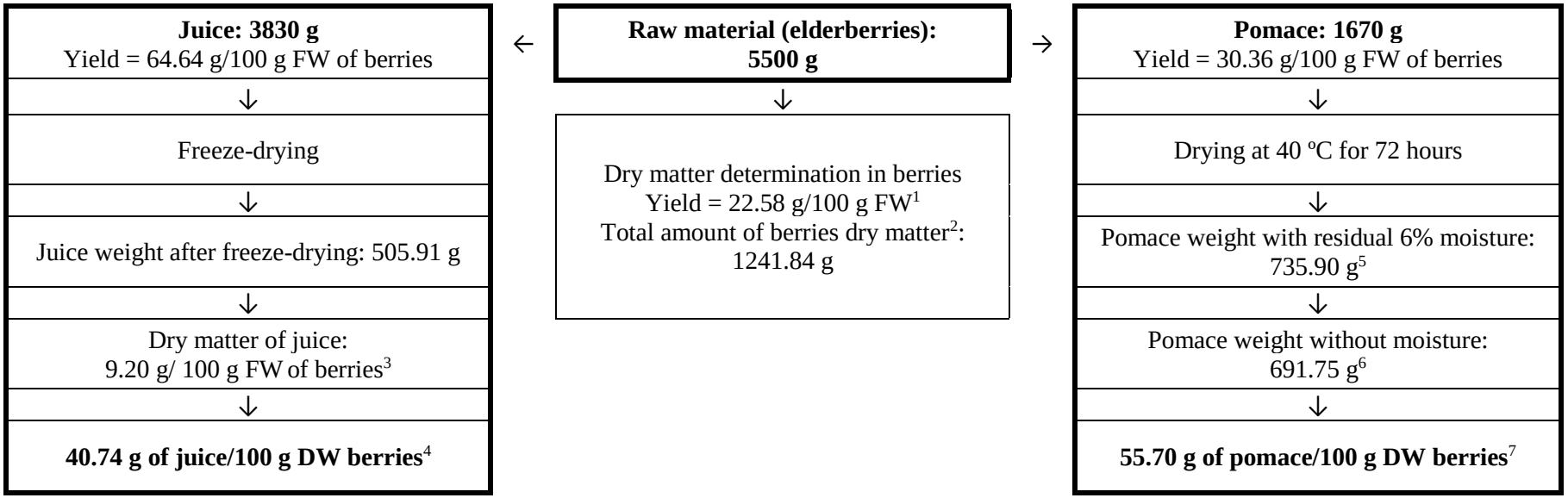
3.1. Chemical parameters of *Sambucus nigra* L. berries and pomace

S. nigra juice was processed from 5.5 kg of thawed berries without branches. The amount of obtained juice was equal to 3.8 kg, while the remaining pomace (peels, pulp residues and seeds) after juice processing amounted 31% of total berries weight (1.7 kg). Seabra et al. (2010) also reported that *S. nigra* pomace amounted of 25% of the total fruit weight [8]. Selected chemical parameters, namely moisture, lipid, protein and mineral contents were determined for pomace and, in comparison berry fruits, were determined prior to the extraction experiments. The results are reported in Table 17. The moisture content of pomace was more than two times lower than berries, 6.18 and 12.72 g/100 g DW, respectively.

Table 17. *S. nigra* fresh berries, pomace and juice chemical parameters and *in vitro* antioxidant capacity

Chemical parameter, g/100 g FW	**Sample**		
	Fresh berries	**Fresh pomace after juice processing**	**Freeze-dried juice**
Moisture content[1*]	77.42	86.62	90.80
Dry matter[1*]	22.58	13.38	9.20
Chemical parameter, g/100 g sample**	**Sample**		
	Berries	**Pomace after juice processing**	**Freeze-dried juice**
Moisture content[2***]	12.72 ± 0.01^{b}	6.18 ± 0.04^{a}	–
Dry matter[2***]	87.28 ± 0.01^{a}	93.82 ± 0.04^{b}	–
Lipid content	15.86 ± 0.05^{a}	15.80 ± 0.12^{a}	–
Nitrogen content	2.13 ± 0.00^{b}	2.11 ± 0.00^{a}	–
Protein content	13.34 ± 0.01^{b}	13.20 ± 0.01^{a}	–
Mineral content	6.61 ± 0.21^{b}	4.11 ± 0.20^{a}	–
***In vitro* antioxidant capacity**	**Berries**	**Pomace after juice processing**	**Freeze-dried juice**
Total phenolic content			
mg GAE/g sample	–	14.70 ± 0.73^{a}	46.15 ± 2.00^{b}
mg GAE/g DW****	35.52 ± 1.69c	8.19 ± 0.41^{a}	25.07 ± 1.09^{b}
$ABTS^{\bullet+}$ scavenging properties			
mg TE/g sample	–	67.02 ± 0.61^{a}	108.26 ± 0.29^{b}
mg TE/g DW	98.25 ± 0.70^{c}	37.33 ± 0.34^{a}	60.30 ± 0.16^{b}
ORAC scavenging properties			
mg TE/g sample	–[na]	–[na]	144.92 ± 3.69
mg TE/g DW	–[na]	–[na]	59.04 ± 1.50

*: Moisture content[1]; dry matter[1]: calculation scheme is given in Figure 14. **Chemical parameters (lipid, total nitrogen, protein, mineral, total phenolic contents and $ABTS^{\bullet+}$ scavenging properties were determined to berries with 12.72% and to pomace with 6.18% residual moisture content. ***Moisture content[2]; dry matter[2]: berries and pomace moisture and dry matter contents were determined after samples drying at 40 °C for 72 hours. ****DW: dry weight; Pomace total phenolic content, $ABTS^{\bullet+}$ and ORAC scavenging properties were calculated: *In vitro* antioxidant capacity of sample × 0.5570 (Figure 13) and juice total phenolic content, $ABTS^{\bullet+}$ and ORAC scavenging properties were calculated: *In vitro* antioxidant capacity of sample × 0.4074 (Figure 13). Referred average values of two determinations ± SD. Different superscript letters in the same row indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).



[1]: 233.80 g FW of berries after drying at 40 °C for 72 hours gave 60.48 g of dried sample (25.87 g/100 g FW), with residual moisture content of 12.72% (Table 17). Recalculated yield of dry matter in berries is 22.58 g/100 g FW.

[2]: 233.80 g FW of berries after drying at 40 °C for 72 hours gave 60.48 g of dried sample, with residual moisture content of 12.72% (Table 17) →

→ $Dry\ matter_{berries} = 60.48 \times \left(\frac{100-12.72}{100}\right) = 52.79$ g; $Dry\ matter_{berries} = \frac{5500 \times 52.79}{233.80} = 1241.85$ g

[3]: $Dry\ matter_{juice} = \frac{505.91}{5500} \times 100 = 9.20\ g/100\ g\ \ FW\ of\ berries$

[4]: $\frac{Dry\ matter_{juice}}{Dry\ matter_{berries}} = \frac{9.20}{22.58} \times 100 = \mathbf{40.74\%}$

[5]: $Pomace\ weight_{with\ 6\%\ moisture} = 1241.85\ g - 505.95 = 735.90\ g$

[6]: $Pomace\ weight_{without\ moisture} = 735.90 \times 0.94 = 691.75\ g$

[7]: $Yield_{pomace} = \frac{691.75}{1241.85} \times 100 = \mathbf{55.70}\ g/100\ g\ DW\ of\ berries$

Figure 14. *Sambucus nigra* L. juice and pomace dry matter calculation scheme

Lipids of berries are accumulated in seeds, pulp and peels, therefore pomace and berries had almost the same lipid content (15.80 and 15.86 g/100 g DW, respectively). Protein content (about 13 g/100 g DW) was also very similar in both samples. Only mineral content was 38% lower in pomace after juice processing than in mature berries.

In the other researchers reports established that nitrogen content of elderberry ranged from 2.7 to 2.9% in berries [24] and mineral content represents 0.9–1.6% of the fresh fruit mass [2].

Based on Wu et al. (2006) and Kaack et al. (2008) dry matter content of *S. nigra* berries range from 18 to 26 g/100 g of fresh weight and according to Skrede et al. (2012) findings suggest that dry matter of elderberry was 11-17 g/100 g of fresh weight [23, 62, 89]. However, Seabra et al. (2010) considerable attention paid to elderberry pomace and attempted to detect target elderberry fractions when pomace dry matter content ranged from 39 to 94%. With respect to differences of *S. nigra* dry matter content in discussed reports, these findings suggest that dry matter content of elderberry depends on drying conditions.

Młynarczyk et al. (2018) indicated that *S. nigra* lipids are mostly concentrated in seeds and lipid content can ranged from 15.9% to 22.4% [2]. Fazio et al. (2013) reported that lipid content in elderberry seeds was 10.5% [9], but Dulf et al. (2013) claimed that lipid content of seeds was 22%. These results could be concluded that *S. nigra* lipid content can vary from 11% to 22% and this conclusion is well agreement with the findings of our work too.

The TPC values measured for *S. nigra* berries, pomace and juice were in the range of 8.19-35.52 mg GAE/g DW of berries (Table 17). Pomace TPC (8.19 mg GAE/g DW) amounted in 23.06% of berries TPC, while juice TPC amounted in 70.58% of berries TPC. Galić et al. (2009) reported that TPC of *S. nigra* berries was 32 mg GAE/g DW and this result is generally comparable with the results of our research work [90]. Mikulic-Petkovsek et al. (2016) presented that elderberry TPC was 6831.1 mg GAE/kg FW, while, in our case, elderberries TPC was 8020.4 mg GAE/kg FW [50].

The $TEAC_{ABTS}$ value of berries was 98.25 mg TE/g DW, while elderberry pomace $ABTS^{\bullet+}$ scavenging properties constituted 37.99% (37.33 mg TE/g DW *versus* 98.25 mg TE/g DW) of a whole berries $ABTS^{\bullet+}$ scavenging properties and juice constituted 61.37% (60.30 mg TE/g DW *versus* 98.25 mg TE/g DW). In addition, elderberry pomace after juice processing, is still valuable source of compounds with *in vitro* antioxidant properties.

3.2. Optimization of SFE-CO_2 parameters for *Sambucus nigra* L. pomace lipophilic fraction

As discussed in chapter 1.5.2, one of the main aspects that should be considered in SFE-CO_2 is the extraction parameters (pressure, time, temperature). The determination of the optimum values for the different variables influencing the SFE-CO_2 efficiency and could increase the recovery of target fractions and compounds, as compared to the conventional extraction methods. Kraujalis and Venskutonis (2013) reported that central composite design (CCD) and response surface methodology (RSM) are commonly applied in combination for SFE-CO_2 optimization. The use of RSM allows the simultaneous graphical optimization of the extraction temperature, pressure and extraction time and the extraction yield can be selected as response variable [78]. Previously, CCD-RSM methodology was successfully employed to optimize SFE-CO_2 for amaranth seeds [78], industrial hemp (*Cannabis sativa* L.) [91], blackcurrant pomace [4], rye bran [92], blackcurrant (*Ribes nigrum* L.) buds [93] and lovage (*Levisticum officinale* Koch.) [94].

In this research work, the main goal of SFE-CO_2 process optimization was to achieve the highest lipophilic fraction yield and recovery from Soxhlet-Hexane extract yield of *S. nigra* pomace after juice processing. The lipophilic fraction yield after Soxhlet extraction with hexane (360 min) amounted 15.8 g/1000 g DW and the recovery from this conventional extraction method was determined in order to compare SFE-CO_2 effectiveness and find out if the traditional method could be changed into environmentally-friendly extraction method for isolation of lipophilic fraction.

For these reasons, pressure (P=15-50 MPa), temperature (T=40-70 °C) and time (τ=30-120 min) were selected as the most important independent variables, on which effectiveness of the whole CO_2 extraction process may depend (Table 18). Response surface model (RSM) was used to optimize the impact of those variables on total lipophilic fraction yield, selected as the response factor. The model consisted of 20 experimental pressure-temperature-time sets, with parameters and results listed in Table 20. It may be observed that selected process parameters had remarkable effects on the selected response: the total yield from pomace varied from 0.10 to 14.65 g/100 g DW and recovery from Soxhlet was equal 0.6-88.15%. Theoretical SFE-CO_2 extract yield varied from 0 g/100 g DW at 15 MPa, 70 °C and 30 min to 14.88 g/100 g DW 32.5 MPa, 40 °C, 75 min. Generally, Central composite design (CCD) and Response-surface method (RSM), experimental and theoretical yields were well as agreement at the same experimental points (Table 18). Experimental SFE-CO_2 non-polar extract yield recovery from Soxhlet extraction equal 0.6-88.2% (Fig. 15).

Table 18. Central composite design matrix (levels of independent variables and variation levels in natural values) for SFE-CO_2 optimization and values of observed responses for the extraction of non-polar constituents from elderberry (*S. nigra*) pomace

No.	SFE-CO_2 parameters			Experimental SFE-CO_2 extract yield, g/100 g DW	Theoretical SFE-CO_2 extract yield, g/100 g DW
	Pressure, MPa	Temperature, °C	Time, min		
1	32.5	40	75	13.96 ± 0.05	14.88
2	15	70	30	0.10 ± 0.03	0
3	15	40	30	3.74 ± 0.01	3.66
4	32.5	55	75	14.14 ± 0.61	14.02
5	32.5	55	75	14.65 ± 0.73	14.02
6	32.5	55	75	14.48 ± 0.11	14.02
7	32.5	55	75	13.62 ± 0.55	14.02
8	15	40	120	6.10 ± 0.12	5.56
9	50	55	75	13.46±0.55	13.32
10	32.5	55	75	13.82 ± 0.55	14.02
11	32.5	55	120	14.64 ± 0.59	14.85
12	15	70	120	0.40 ± 0.04	0.67
13	32.5	55	75	14.13 ± 0.55	14.02
14	50	70	30	13.77 ± 0.19	14.22
15	50	40	30	13.23±0.73	12.86
16	32.5	70	75	13.67 ± 0.28	13.11
17	50	40	120	13.73 ± 0.19	13.80
18	50	70	120	13.98±0.55	13.97
19	15	55	75	1.57 ± 0.13	2.01
20	32.5	55	30	13.88 ± 0.88	14.03
Optimal conditions:					
	35	**53**	**105**	**14.05 ± 0.2**	15.20

The adequacy of the model, as it may be judged from the total determination coefficient R^2 (0.9942), indicates reasonable fit of the models to the experimental data. Model analysis also showed good agreement between the adjusted and predicted coefficients of determination (R^2): 0.9890 and 0.9549, respectively. Calculated adequate precision (Press) values of 38.190, which compare the range of the predicted values to the average prediction error at the experimental design points (desirable signal to noise ratio > 4), indicate that the signal is adequate, and the model can be used to navigate the design space. Quadratic regression model analysis for SFE-CO_2 yield (Table 19) showed that the model was significant according to the Student test ($p < 0.05$) with a calculated *F*-value of 191.18 and *lack of fit* was not significant relative to the pure error ($p = 0.1210$).

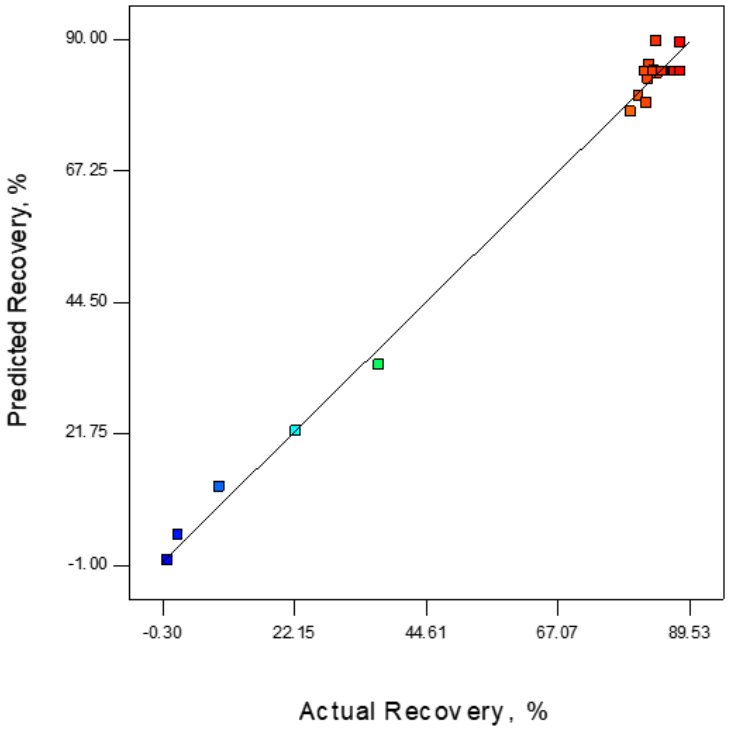


Figure 15. *S. nigra* pomace actual and predicted recovery (%) from Soxhlet after SFE-CO_2

Table 19. Analysis of variance of the regression parameters for response surface quadratic model for SFE-CO_2 extract yields

Source	SS	df	MS	F-value	p-value
Model	525.99	9	58.44	191.18	< 0.0001*
P-MPa	316.4	1	316.40	1035.01	< 0.0001*
T-temperature	7.82	1	7.82	25.59	0.0005*
τ-time	1.71	1	1.71	5.59	0.0397*
PT	12.83	1	12.83	41.96	< 0.0001*
Pτ	0.4704	1	0.47	1.54	0.2431**
Tτ	0.6929	1	0.69	2.27	0.1631**
P^2	110.11	1	110.11	360.2	< 0.0001*
T^2	0.0017	1	0.0017	0.0056	0.9418**
τ^2	0.4819	1	0.48	1.58	0.2378**
Residual	3.06	10	0.31		
Lack of Fit	2.31	5	0.46	3.08	0.121
Pure Error	0.7486	5	0.15		
Cor Total	529.04	19			

*: significant; **: not significant; SS: sum of square; df: degree of freedom; MS: mean square; F: Fisher value.

The results in Table 3 show that P, T, τ, PT interaction and second-order term of P^2 were significant on the total SFE-CO_2 extract yield in the following order: P ($p < 0.0001$, $F=1035.01$) > P^2 ($p < 0.0001$, $F=360.20$) > PT ($p < 0.0001$) > T ($p = 0.0005$) > τ ($p = 0.0397$). Second order

polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is given in the equation (1).

$$Yield_{SFE-CO_2} = 14.02 + 5.62 \times P - 0.88 \times T + 0.41 \times \tau + 1.27 \times (PT) - 0.24 \times (P\tau) - 0.29 \times (T\tau) - 6.33 \times P^2 - 0.025 \times T^2 + 0.42 \times \tau^2 \quad (1)$$

Response surface plots showing the effect on three independent variables: A (effect of temperature and pressure), B (effect of time and pressure) and C (effect of temperature and time), on *S. nigra* pomace SFE-CO_2 yields are shown in Figures 16-18. It follows from Figure 14, that at the P=15 MPa and T=70 °C obtained SFE-CO_2 extract yield was the lowest and further, T=40 °C resulted in extract yield increasing at the same pressure. It may be explained by the remarkable decrease of CO_2 density at lower pressure levels by increasing temperature, resulting in a weaker diffusivity and solvating power [95]. Figure 14 reflected that increasing of pressure from 20 MPa to 30 MPa equal in increased SFE-CO_2 extract yield (10.38-13.01 g/100 g pomace) and further increasing of pressure guarantee the highest non-polar extract yields.

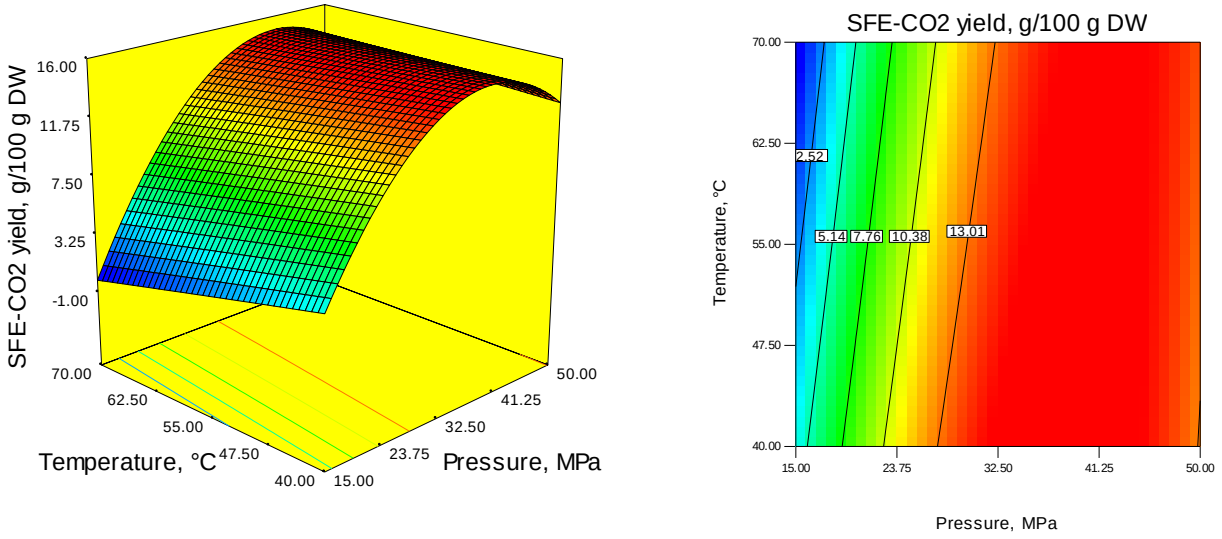


Figure 16. Response surface 3D and 2D plots showing the effects of independent P variable, effect of temperature and pressure ($\tau_{const.}$=75 min) on SFE-CO_2 yields (g/100 g pomace)

Based on the data presented in Figure 15 the extraction pressure had important impact on SFE-CO_2 extract yields and increasing of extraction pressure from ~30 MPa till ~49 MPa (τ=75 min) equal to the highest yields (13.6-16 g/100 g DW) and the extraction time up to 97.5 min also related to increased SFE-CO_2 yields. Figure 16 illustrated that SFE-CO_2 temperature from ~42 °C to 53 °C and extraction time up to 97.5 min, when P=32.5 MPa, resulted in the highest extraction yields 14-16 g/100 g pomace.

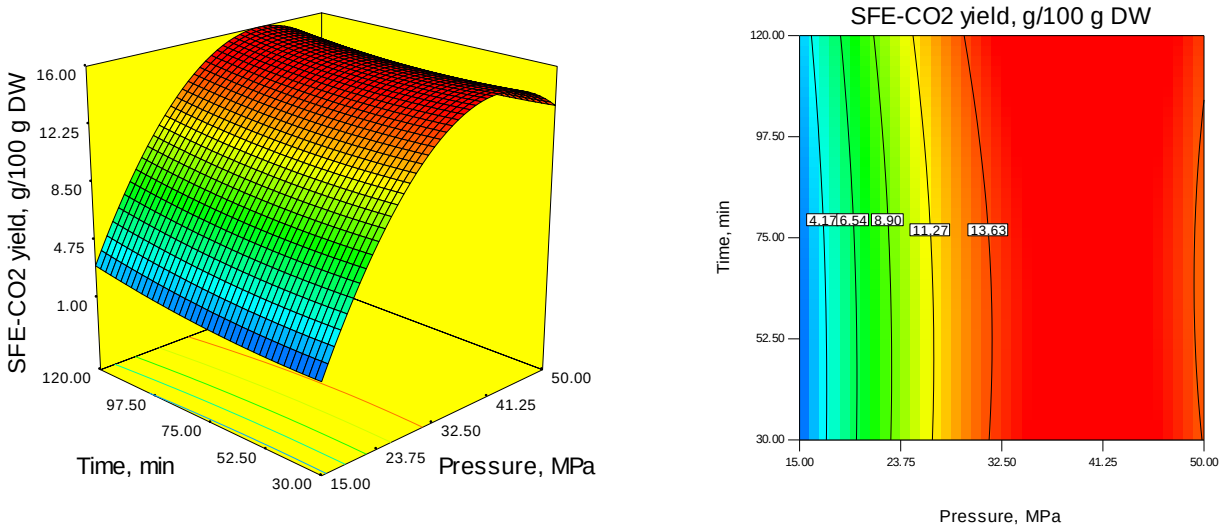


Figure 17. Response surface 3D and 2D plots showing the effects of time and pressure ($T_{const.}$ =55°C) on SFE-CO_2 yields (g/100 g pomace)

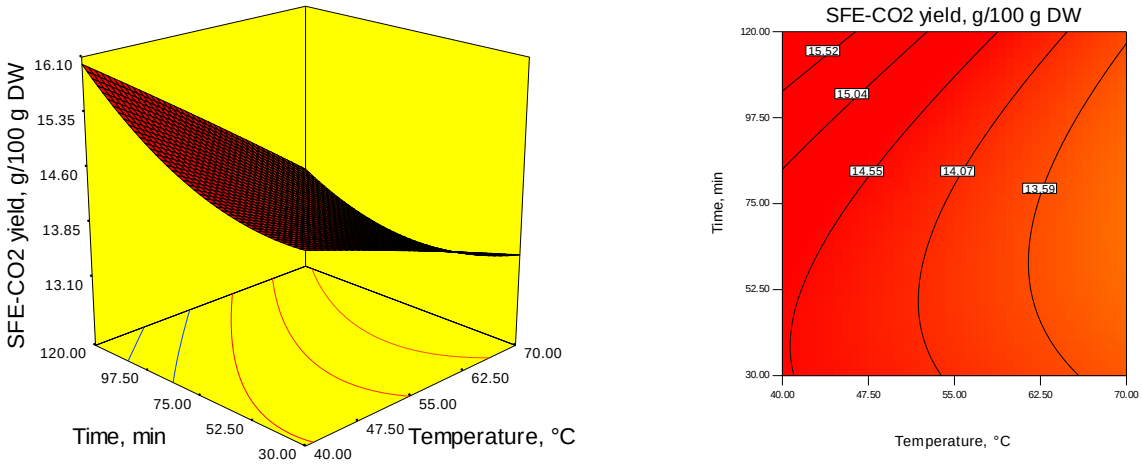


Figure 18. Response surface 3D and 2D plots showing the effects of temperature and time ($P_{const.}$=32.5 MPa) on SFE-CO_2 yields (g/100 g pomace)

Considering all responses, the following optimal SFE-CO_2 conditions were selected: P=35 MPa; T=53 °C; τ=105 min, while the pressure was the most important independent variable and during this extraction we need 39 kg CO_2/kg* elderberry pomace.

$*$: 1) $CO_2\ density\ (normal\ temperature\ and\ pressure: 20℃, 1\ atm) = 1.842\ kg/m^3 = 1.842 \times 10^{-3}\ kg/l$;
2) $Flow\ rate: 2\ SL/min = 2 \times 1.842 \times 10^{-3} = 3.684 \times 10^{-3}\ kg/min$;
3) $Extraction\ time: 105\ \text{min} \rightarrow 105 \times 3.684 \times 10^{-3} = 0.39\ kg\ CO_2$;
4) $CO_2\ kg/kg\ sample = 0.39\ kg\ CO_2/0.01\ kg\ elderberry\ pomace = 39$

However, to the best of our knowledge tour study, little information is so far available on elderberry pomace and in the study of Seabra et al. (2010), SFE-CO_2 conditions were not optimized for lipophilic fraction isolation from elderberry pomace. In this researchers' study SFE-CO_2 parameters, 40 °C and 20 MPa, were selected based on the literature information, regarding the solubility of the main constituents from fruits non-polar fraction in supercritical CO_2 and on the anthocyanin stability [8].

3.3. Efficiency of conventional and innovative extraction techniques for target fraction isolation from *S. nigra* pomace

3.3.1. Isolation of non-polar constituents

S. nigra pomace were extracted by using five different extraction methods, out of which two were conventional (Soxhlet, SLE), two – high pressure (SFE-CO_2, PLE) and the last one was ultrasound-assisted extraction method. The results of these extractions and extraction parameters are presented in Table 20.

The highest lipophilic fraction yield (19.86 g/100 g pomace) was obtained after ultrasound-assisted extraction, while the lowest extraction yield (13.47 g/100 g pomace) – after solid-liquid extraction. In addition, it should be noted that UAE yielded 15 and 47% higher amounts of hexane extract in 18-fold shorter extraction time (20 *versus* 360 min), as compared to conventional Soxhlet and SLE methods, respectively. UAE kinetics are given in Figure 19: the major portion (83 %) of non-polar extract was obtained after the first five minutes of UAE with hexane (16.40 g/100 g pomace), while additional 15 min contributed to the remaining 17 % (3.46 g/100 g pomace) of non-polar extract.

Table 20. Conventional, high pressure and ultrasound-assisted extractions parameters and isolated non-polar constituents (Soxhlet, SLE, SFE-CO_2, PLE, UAE) yields

Sample	Particle size, mm	Extraction parameters			Yield
		Pressure, MPa	Temperature, ˚C	Time, min	g/100g pomace*
Conventional extraction methods					
Soxhlet-He	0.5	0.1**	69	360	15.80 ± 0.12^{c}
SLE-He	0.5	0.1	60	360	13.47 ± 0.07 ^{a}
High pressure and ultrasound-assisted extraction methods					
SFE-CO_2	0.5	35	53	105	14.05 ± 0.20 ^{b}
PLE-He-1	0.5	10	60	15	16.41 ± 0.06 ^{d}
PLE-He-2	0.5	10	60	30	15.85 ± 0.19 ^{cd}
UAE-He	0.5	0.1	50	20	19.86 ± 0.16 ^{e}

*: pomace dry matter 94%; *: atmospheric pressure: 0.1 MPa. Average values of two determinations ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

Evaluating the efficiency of high-pressure extraction techniques, PLE duration (15 or 30 min) did not significantly affect the extraction yield. On average, 16.10 g/100 g DW of lipophilic constituents were obtained after PLE, showing 81% recovery as compared to the most efficient technique UAE. It should be noted that PLE can shorten extraction time up to 24-fold (15 *versus* 360 min) and increase the yield of hexane-soluble constituents by 2-16%, as compares to conventional extraction methods.

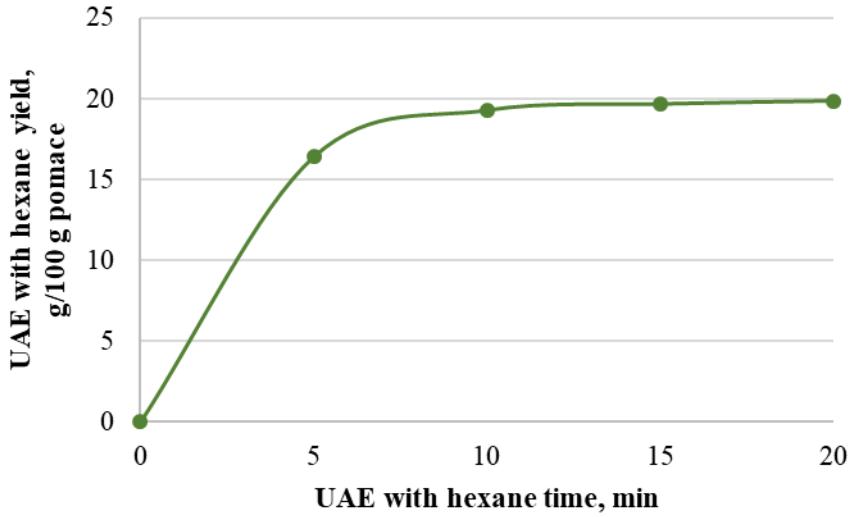


Figure 19. Elderberry pomace kinetics during ultrasound-assisted extraction with hexane

Another high-pressure extraction method SFE-CO_2 under optimized extraction conditions yielded 14.05 g/100 g pomace of elderberry pomace lipophilic constituents, showing 71-104% extraction efficiency as compared to other extraction methods tested. It should be noted that SFE-CO_2 in 3.4-fold shorter extraction time (105 *versus* 360 min) amounted 89% and 104% of the total Soxhlet and SLE yields. The yields of PLE and UAE under 3.5-7-fold lower extraction times were significantly higher as compared to SFE-CO_2. However, the latter technique offers an advantage of using environment-friendly and food-grade solvent CO_2, as compared to hexane-extraction based techniques, and does not require solvent removal after extraction (according to Directive 2009/32/EC, maximum hexane residue limits in the extracted foodstuff can be 1mg/kg [96]). Therefore, SFE-CO_2 could be regarded as promising technique for lipophilic constituent isolation form elderberry pomace.

Seabra et al. (2010) also explored the potential of conventional (SLE) and high-pressure extraction methods (SFE-CO_2 and PLE) for elderberry pomace valorisation. In this work is was noticed that the SFE-CO_2 extraction (15 min static and 40 min dynamic extraction period; 20 MPa and 40 °C temperature) yields depends on pomace humidity and the lower moisture content can

guarantee higher extraction yield: pomace humidity 61% resulted in 2.8 g/100 g DW, while pomace humidity 6.1%, resulted in about 17 g/100 g DW [8]. These reported results are generally comparable with the results of our research work.

3.3.2. Isolation of polar constituents

S. nigra pomace and solid residues after SFE-CO_2 were extracted by ethanol and water to isolate polar constituents. Conventional (Soxhlet, SLE), high pressure (PLE) and ultrasound-assisted extraction methods were used for this purpose. All results and extractions parameters are shown in Table 21. The highest polar extract yield (21.85 g/100 g pomace) was obtained after pressurized-liquid extraction with water (30 min), while the lowest extraction yields (2.00 and 2.71 g/100 g pomace without significant differences) – after solid-liquid and Soxhlet extractions with acetone, respectively. Further, it should be noted that, as compared to conventional Soxhlet and SLE methods, UAE yielded 45-48% higher amounts of ethanol extract (12.88 g/100 g pomace *versus* 6.69 and 7.05 g/100 g pomace, respectively) in up to 18-fold shorter extraction time (20 *versus* 360 min).

Table 21. Conventional (Soxhlet, SLE), high pressure (PLE) and ultrasound-assisted extractions (UAE) parameters and isolated polar constituents' yields

Extraction	Extraction parameters				Yield	Yield
	Particle size, mm	*Pressure, MPa*	*Temperature, ˚C*	*Time, min*	*g/100 g*	*g/100 g pomace**
Polar extract from starting material Conventional extraction methods						
Soxhlet-He-Ac	0.5	0.1**	50	360	3.25 ± 0.04^{a}	2.71 ± 0.03^{a}
Soxhlet-He-Ac-EtOH	0.5	0.1	70	360	8.29 ± 0.01^{b}	6.69 ± 0.16^{b}
SLE-He-Ac	0.5	0.1	40	360	2.40 ± 0.16^{a}	2.00 ± 0.14^{a}
SLE-He-Ac-EtOH	0.5	0.1	60	360	8.68 ± 0.03^{b}	7.05 ± 0.06^{b}
SLE-He-Ac-EtOH-H_2O	0.5	0.1	60	360	12.19 ± 0.59^{c}	9.45 ± 0.45^{d}
Polar extracts from solid residue after SFE-CO_2 at o.c. (35 MPa, 53 °C, 105 min); High pressure and ultrasound-assisted extraction methods						
PLE-EtOH-1	0.5	10	60	15	9.51 ± 0.32^{b}	8.17 ± 0.28^{c}
PLE-EtOH-2	0.5	10	60	30	8.63 ± 0.19^{b}	7.42 ± 0.17^{bc}
PLE-EtOH-1-H_2O	0.5	10	140	15	15.44 ± 0.73^{d}	9.67 ± 0.40^{d}
PLE-H_2O-1	0.5	10	140	15	14.67 ± 0.02^{d}	12.61 ± 0.02^{e}
PLE-H_2O-2	0.5	10	140	30	25.43 ± 0.23^{e}	21.85 ± 0.20^{f}
UAE-EtOH	0.5	0.1	40	20	14.99 ± 0.12^{d}	12.88 ± 0.15^{e}
UAE-EtOH-H_2O	0.5	0.1	40	20	8.94 ± 0.27^{b}	7.68 ± 0.23^{bc}

*: pomace dry matter 94%; *: atmospheric pressure: 0.1 MPa. Average values of two determinations ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

Evaluating the effectiveness of pressurized-liquid extraction method, PLE time (15 or 30 min) did not significantly affect the extraction yields. On average, 7.8 g/100 g pomace of polar

constituents were obtained after PLE with ethanol, showing on average 114% recovery as compared to the traditional extraction methods (Soxhlet and SLE). In addition, it should be noted that PLE can not only increase the yield of ethanol-soluble constituents, but also can shorten extraction time even up to 24-fold (15 *versus* 360 min). Further, PLE with H_2O duration (15 or 30 min) significantly effect extraction yield and after 30 min of extraction the water-soluble constituents yield was 2-fold higher (12.61 *versus* 21.85 g/100 g pomace). There are no significant differences among extraction yields of conventional SLE with H_2O and PLE with H_2O (with solid residues after extraction with EtOH), 9.45 and 9.67 g/100 g pomace, respectively, but the PLE time was up to 24-fold shorter (15 *versus* 360 min).

Another innovative extraction method, UAE with H_2O (with solid residues after extraction with EtOH) yielded 7.7 g/100 g pomace of elderberry pomace polar constituents, showing 81% extraction efficiency as compared to conventional SLE method tested. In addition, it should be remarked that UAE in 18-fold shorter extraction time (20 min *versus* 360 min) amounted 81.3% of the total SLE with H_2O yield.

According to the results of other researchers, the elderberry pomace polar constituents' yield after PLE with EtOH could be about 13 g/100 g DW with 45 min extraction time, which is generally lower as compared to the results of our study. However, PLE with H_2O yield was reported to amount ~ 18 g/100 g DW [8] and this result is 18 % lower than the yield of corresponding fraction from our work. Differences between these results (13 g/100 g DW versus 7.8 g/100 g DW and 18 g/100 g DW *versus* 21.85 g/100 g DW) could be explained by different extraction times and temperatures (45 min *versus* 30 min and 40 °C *versus* 140 °C, respectively) [8]. The conclusions of other reports confirmed that UAE can also offer higher yield of polar constituents in shorter times, reduced volume of solvents and lower energy requirements. Furthermore, Oniszczuk et al. (2016) did not find any evidence that ultrasound-assisted extraction can cause polyphenols degradation [42].

3.3.3. Development of multi-step extraction schemes for non-polar and polar fractions isolation from S. nigra pomace

S. nigra pomace non-polar and polar fractions were isolated by using multi-step extractions, out of which two were conventional (Soxhlet, SLE) and six – non-conventional multi-step extraction methods. The proposed multi-step extraction techniques are presented in Figure 23. Evaluating the efficiency of multi-step extractions techniques, the major factors were: total non-polar and polar fraction yield, non-polar and polar fraction ratio, total multi-step extraction time and whether

solvents were needed. The highest multi-step extraction yield (35.90 g/100 g pomace) was obtained after SFE-CO_2, followed by PLE-H_2O-2 (Scheme Nr.8), while the lowest extraction yield (21.47 g/100 g pomace) – after SFE-CO_2, followed by PLE-EtOH-2 (Scheme Nr.6). The multi-step SFE-CO_2+PLE-H_2O-2 extraction lasted 135 min, while SFE-CO_2 duration was 105 min and PLE-H_2O-2 – 30 min. Though, according to the kinetics of SFE-CO_2 (Fig.20), extraction duration could be shortened till 30 min. In addition, SFE-CO_2 kinetics indicated, that the major portion (98%) of non-polar extract was obtained after 30 minutes of SFE-CO_2 (13.82 g/100 g pomace), while additional 90 min contributed to the remaining 2.3% (0.33 g/100 g pomace) of non-polar extract. In addition, during this extraction we need up to 3.5-fold lower CO_2 kg/kg elderberry pomace as compared to 105 min SFE-CO_2 extraction (39 kg CO_2 /kg elderberry pomace *versus* 11 kg CO_2 /kg elderberry pomace):

$$1)\ Extraction\ time: 30\ \text{min} \rightarrow 30 \times 3.684 \times 10^{-3} = 0.11\ kg\ CO_{2;}$$

$$2)\ CO_2\ kg/kg\ sample = 0.11\ kg\ CO_2/0.01\ kg\ elderberry\ pomace = 11$$

In this case, SFE-CO_2+PLE-H_2O-2 extraction duration could be 60 min. Non-polar and polar fractions (N/P) ratio was obtained 0.6 (39% non-polar and 61% polar fraction). However, after the SFE-CO_2+PLE-EtOH-2 extraction, when was obtained the lowest extraction yield, non-polar and polar fraction ratio was the highest (1.9: 65% non-polar/35% polar fraction).

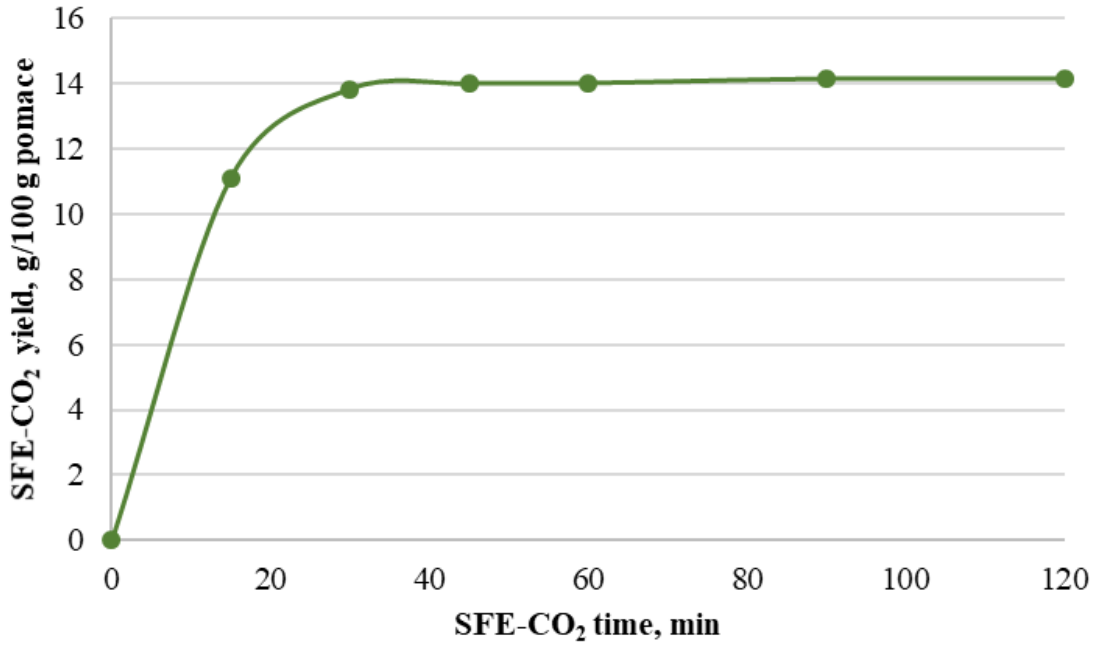


Figure 20. The kinetics of *S. nigra* pomace SFE-CO_2 extraction at 32.5 MPa, 55 °C

The second highest multi-step extraction yield (34.61 g/100 g pomace) was obtained after SFE-CO_2, followed by UAE with ethanol and UAE with water. This extraction lasted 145 min, but, as mentioned before, SFE-CO_2 extraction could be shortened till 30 min, and, in this case, extraction time could be 70 min. UAE with EtOH kinetics are given in Figure 21. This figure reflects that the major portion (82%) of polar extract was obtained after the first ten minutes of UAE with ethanol (10.6 g/100 g pomace), while additional 10 min contributed to the remaining 18% (2.28 g/100 g pomace) of elderberry polar extract. UAE with H_2O kinetics are given in Figure 22 and it showed, that the major portion (87 %) of polar extract was obtained after the first five minutes (6.66 g/100 g pomace), while additional 15 min contributed to the remaining 13% (1.02 g/100 g pomace) of polar extract. The SFE-CO_2+UAE with three extraction steps and with ethanol-based extraction in the second step, amounted in 3.6% lower yield, as compared to SFE-CO_2+PLE-H_2O-2 (34.61 g/100 g *versus* 35.90 g/100 g). Therefore, proposed extraction Scheme Nr.8 (SFE-CO_2+PLE-H_2O-2) had the major two advantages: the highest total extraction yield and it was environmental-friendly, while the drawback is quite expensive extraction equipment.

Further, it should be noted, that SFE-CO_2+PLE-H_2O-2 extraction yielded in 10.9% higher amount of total extract (31.97 *versus* 35.90 g/100 g pomace), in up to 10.6-fold shorter multi-step extraction time (1440 min *versus* 135 min), as compared to conventional SLE extraction (Scheme Nr.2). Multi-step extraction Scheme Nr.7 (SFE-CO_2+PLE-H_2O-1) also suggested environmental-friendly way to isolate non-polar and polar fractions from elderberry pomace with the 26.66 g/100 g yield, during 120 min, while conventional Soxhlet multi-step extraction amounted in 5.5% lower yield (25.20 g/100 g *versus* 26.66 g/100 g) and in 9-fold longer time (1080 min *versus* 120 min).

The lowest multi-step extraction yields (22.22 g/100 g pomace and 21.47 g/100 g pomace) were obtained after SFE-CO_2, followed by PLE-EtOH (15 min and 30 min, respectively). In addition, these multi-step extractions yielded 13.3-31.7% lower amounts of total extract in, on average, 8.1-10.8-fold shorter total extraction time as compared with conventional Soxhlet and SLE multi-step extractions.

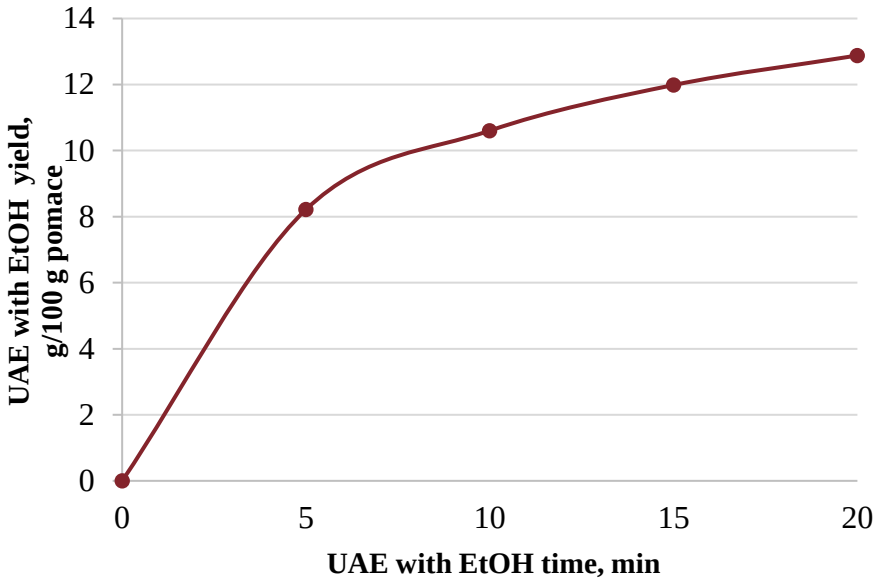


Figure 21. *S. nigra* solid residue after SFE-CO_2 at o.c. (35 MPa, 53 °C, 105 min) kinetics during ultrasound-assisted extraction with ethanol

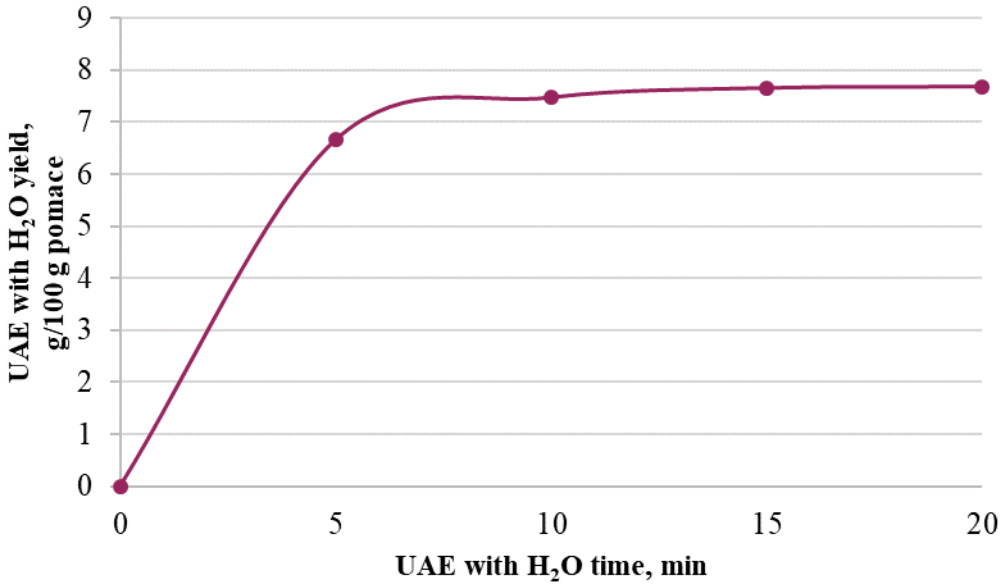


Figure 22. *S. nigra* solid residue after SFE-CO_2 at o.c. (35 MPa, 53 °C, 105 min) kinetics during ultrasound-assisted extraction with H_2O

Proposed extraction schemes for isolation non-polar and polar fractions from *S. nigra* pomace powder after juice processing

↓

SCHEME NR. 1 **Soxhlet extraction**	**SCHEME NR. 2** **Solid-liquid extraction (SLE)**	**SCHEME NR. 3** **SFE-CO_2+PLE**	**SCHEME NR. 4** **SFE-CO_2+UAE**
1. Soxhlet-He (15.80 g/100g) + 2. Soxhlet-He-Ac (2.71 g/100g) + 3. Soxhlet-He-Ac-EtOH (6.69 g/100g)	1. SLE-He (13.47g/100g) + 2. SLE-He-Ac (2.00 g/100g) + 3. SLE-He-Ac-EtOH (7.05 g/100g) + 4. SLE-He-Ac-EtOH-H_2O (9.45 g/100g)	1. SFE-CO_2 (14.05 g/100g) + 2. PLE-EtOH-1*** (8.17 g/100g) + 3. PLE-EtOH-1-H_2O (9.67 g/100g)	1. SFE-CO_2 (14.05 g/100g) + 2. UAE-EtOH (12.88 g/100g) + 3. UAE-EtOH-H_2O (7.68 g/100g)
τ*: 1080 min (360 min x 3) **∑pomace: 25.20 g/100g**	**τ: 1440 min (360 min x 4)** **∑pomace: 31.97 g/100g**	**τ: 135 min (105 min_1+15 min_2+15 min_3)** **∑pomace: 31.89 g/100g**	**τ: 145 min (105 min_1+20 min_2+20 min_3)** **∑pomace: 34.61 g/100g**
63% non-polar fraction 37% polar fraction **N/P ratio**: 1.7**	42% non-polar; 58% polar fraction **N/P ratio: 0.7**	44% non-polar fraction 56% polar fraction **N/P ratio: 0.8**	41% non-polar fraction 59% polar fraction **N/P ratio: 0.7**

SCHEME NR. 5 **SFE-CO_2+PLE-EtOH-1*** **	**SCHEME NR. 6** **SFE-CO_2+PLE-EtOH-2*** **	**SCHEME NR. 7** **SFE-CO_2+PLE-H_2O-1*** **	**SCHEME NR. 8** **SFE-CO_2+PLE-H_2O-2*** **
1. SFE-CO_2 (14.05 g/100g) + 2. PLE-EtOH-1 (8.17 g/100g)	1. SFE-CO_2 (14.05 g/100g) + 2. PLE-EtOH-2 (7.42 g/100g)	1. SFE-CO_2 (14.05 g/100g) + 2. PLE-H_2O-1 (12.61 g/100g)	1. SFE-CO_2 (14.05 g/100g) + 2. PLE-H_2O-2 (21.85 g/100g)
τ: 120 min (105 min_1+15 min_2) **∑pomace: 22.22 g/100g**	**τ: 145 min (105 min_1+30 min_2)** **∑pomace: 21.47 g/100g**	**τ: 120 min (105 min_1+15 min_2)** **∑pomace: 26.66 g/100g**	**τ: 135 min (105 min_1+30 min_2)** **∑pomace: 35.90 g/100g**
63% non-polar fraction 37% polar fraction **N/P ratio: 1.7**	65% non-polar fraction 35% polar fraction **N/P ratio: 1.9**	53% non-polar fraction 47% polar fraction **N/P ratio: 1.1**	39% non-polar fraction 61% polar fraction **N/P ratio: 0.6**

*τ: total extraction time duration; **N/P ratio: non-polar fraction yield/polar fraction yield;

***: PLE-EtOH-1 and PLE-H_2O-1 duration 15 min; PLE-EtOH-2 PLE-H_2O-2 duration 30 min.

Figure 23. Proposed multi-step extraction schemes for isolation non-polar and polar fractions from *S. nigra* pomace powder after juice processing

Thus, proposed multi-step extraction scheme is Scheme Nr.8 (SFE-CO2+PLE-H_2O-1). This multi-step extraction offers an advantage of using environment-friendly and food-grade solvents as CO_2 (in the first extraction step) and H_2O (in the second extraction step), as compared to hexane, acetone, ethanol-extraction based techniques, and does not require solvent removal after extractions (while hexane residue limits in the foodstuff can be 1 mg/kg; ethanol and acetone residues cannot be)[96].

3.4. Antioxidant activity assessment of extracts and solid residues

3.4.1. Total phenolic content (TPC) by Folin-Ciocalteu's assay

Antioxidants can deactivate radicals by two major mechanisms, HAT and SET. HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound [82].

The Folin–Ciocalteu assay is well-known method for determination of total phenolic content (TPC) and it is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds under alkaline condition. The Folin–Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric *in vitro* assay of phenolic and polyphenolic antioxidants. Polyphenols in plant extracts react with redox Folin-Ciocalteu reagent to form a blue complex that can be quantified by visible-light spectrophotometry. Gallic acid is the commonly used reference standard and results of TPC is usually expressed as gallic acid equivalents [82, 97].

Total phenolic contents of non-polar and polar extracts are shown in Table 22 and 23, respectively. The highest total phenolic contents were obtained to extracts after conventional Soxhlet and solid-liquid extractions, 4.7 mg and 2.9 GAE/g pomace, respectively, while lower TPC, 1.83-2.44 mg GAE/g pomace, had non-polar extracts after innovative extractions. Evaluating the TPC after high-pressure extraction techniques, SFE-CO_2 duration was up to 3.4-fold shorter (360 *versus* 105 min) and TPC was 17-48% lower as compared to other conventional (SLE and Soxhlet, respectively) extraction methods. TPC of non-polar extract after PLE and UAE were 1.83 mg GAE/g pomace and it should be noticed that recovery from the highest TPC (4.69 mg GAE/g pomace) was 39%, but extraction times were up to 16-fold shorter than conventional extraction methods (15 min (PLE-1), 20 min (UAE), 30 min (PLE-2) *versus* 360 min). The highest TPC of extract (29.66 mg GAE/g extract) had non-polar extract after Soxhlet. In comparison of the mentioned highest result, TPC of extract after UAE was 20% lower (23.8 *versus* 29.7 mg GAE/g

extract), but UAE duration was 18-fold shorter than Soxhlet extraction. SFE-CO_2 extract amounted in 17.4 mg GAE/g extract and it is 1.7-fold lower result as compared to TPC of Soxhlet extract, but SFE-CO_2 lasted 3.4-fold shorter (360 min *versus* 105 min). These results could be concluded that SFE-CO_2 was the most effective extraction method from non-conventional extraction techniques with the highest TPC yield (17.4 mg GAE/g extract and 2.4 mg GAE/g pomace). Further, the extraction time had impact on TPC of non-polar extracts and longer extraction duration resulted in higher TPC.

Table 22. Total phenolic content of *S. nigra* non-polar extracts after conventional, high pressure and ultrasound-assisted extractions

Non-polar extract from starting material	Extraction conditions	TPC, mg GAE/g extract	TPC, mg GAE/g pomace*
Conventional extraction methods			
Soxhlet-He	69 °C; 360 min	29.66 ± 0.29^{d}	4.69 ± 0.05^{d}
SLE-He	60 °C; 360 min	21.83 ± 0.11^{c}	2.94 ± 0.01^{c}
High pressure and ultrasound-assisted extraction methods			
SFE-CO_2	35 Mpa; 53 °C; 105 min	17.36 ± 0.62^{b}	2.44 ± 0.09^{b}
PLE-He-1	10 Mpa; 60 °C; 15 min	11.15 ± 0.40^{a}	1.83 ± 0.07^{a}
PLE-He-2	10 Mpa; 60 °C; 30 min	12.84 ± 0.62^{a}	1.83 ± 0.08^{a}
UAE-He	40 °C; 20 min	23.75 ± 1.87^{c}	1.83 ± 0.09^{a}

*mg GAE/g pomace: TPC, mg GAE/g extract were recalculated into TPC, mg GAE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

Table 23. Total phenolic content of *S. nigra* polar extracts after conventional, high pressure and ultrasound-assisted extractions

Polar extract	Extraction conditions	TPC, mg GAE/g extract	TPC, mg GAE/g pomace*
Polar extract from starting material Conventional extraction methods			
Soxhlet-He-Ac	80 °C; 360 min	41.63 ± 2.35^{a}	1.13 ± 0.05^{a}
Soxhlet-He-Ac-EtOH	80 °C; 360 min	43.80 ± 0.19^{a}	2.93 ± 0.01^{b}
SLE-He-Ac	40 °C; 360 min	40.72 ± 2.35^{a}	0.81 ± 0.05^{a}
SLE-He-Ac-EtOH	60 °C; 360 min	60.43 ± 1.31^{b}	4.26 ± 0.09^{c}
SLE-He-Ac-EtOH-H_2O	40 °C; 360 min	116.44 ± 4.11^{e}	11.00 ± 0.39^{h}
Polar extract from solid residue after SFE-CO2; High pressure and ultrasound-assisted extraction methods			
PLE-EtOH-1	10 Mpa; 60 °C; 15 min	93.37 ± 1.11^{d}	7.63 ± 0.08^{ef}
PLE-EtOH-2	10 Mpa; 60 °C; 30 min	97.69 ± 3.35^{d}	7.24 ± 0.24^{e}
PLE-EtOH1-H_2O	10 Mpa; 40 °C; 15 min	61.25 ± 2.34^{b}	5.92 ± 0.23^{d}
PLE-H_2O-1	10 Mpa; 40 °C; 15 min	65.39 ± 0.80^{b}	8.24 ± 0.10^{f}
PLE-H_2O-2	10 Mpa; 40 °C; 30 min	116.44 ± 4.11^{e}	25.44 ± 0.90^{i}
UAE-EtOH	40 °C; 20 min	76.92 ± 0.59^{c}	9.91 ± 0.07^{g}
UAE-EtOH-H_2O	40 °C; 20 min	119.52 ± 0.80^{e}	9.19 ± 0.38^{g}

*mg GAE/g pomace: TPC, mg GAE/g extract were recalculated into TPC, mg GAE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

The TPC values measured for polar elderberry extracts were in the range of 40.72-119.52 mg GAE/g extract and in the range of 0.81-25.44 mg GAE/g pomace (Table 23). The highest values were determined for water extracts, followed by ethanol and acetone extracts, whereas water fractions after UAE (119.5 mg GAE/g extract), PLE (30 min) (116.4 mg GAE/g extract) and SLE (116 mg GAE/g extract) contained the highest TPC. The amount of phenolics, which is extracted by different solvents from 1 g of pomace is also presented and, in this case, water fraction after PLE (30 min) provides the highest yield of TPC, 25.44 mg GAE/g pomace, followed by 11.00 mg GAE/g pomace of water fraction after SLE. In additional, it should be discussed that mentioned SLE lasted 12-fold longer as compared to PLE (360 min *versus* 30 min). The significant differences and extraction time impact were noticed of TPC after PLE with water, which took 15 min and 30 min with TPC results 8.24 and 25.44 mg GAE/g pomace, while PLE with water (after PLE with EtOH), yielded in 4.3-fold lower TPC (5.92 versus 25.44 mg GAE/g pomace). Further, TPC values for PLE ethanol extracts were in range of 7.24-7.63 mg/g GAE pomace without significant differences, so the extraction time impact on TPC was not determined and PLE with ethanol extraction could be shorten till 15 min. Evaluating the efficiency of innovative extraction methods, it should be noted that TPC in UAE ethanol extract was 25% higher than in PLE ethanol extract (7.4 *versus* 9.9 mg GAE/g pomace) and TPC in UAE hydrophilic extract was 17% higher than in PLE (15 min) water extract (9.19 *versus* 8.24 mg GAE/g pomace), but was 64% lower than in PLE (30 min) hydrophilic extract (9.19 *versus* 25.44 mg GAE/g pomace). The lowest TPC values were determined for SLE semi-polar acetone extract (0.81 mg GAE/g pomace), followed by Soxhlet acetone extract (1.13 mg GAE/g pomace), Soxhlet ethanol extract (2.93 mg GAE/g pomace) and SLE ethanol extract (4.26 mg GAE/g pomace). As compared innovative and conventional extraction methods, latest fractions contained the lowest TPC: Soxhlet ethanol fraction TPC was 59.5-70.4% lower than TPC of PLE and UAE ethanol fractions (2.93 *versus* 7.24 mg GAE/g pomace and 2.93 *versus* 70.4 mg GAE/g pomace); TPC of SLE ethanol extract was 44.2-70% lower than TPC of PLE and UAE ethanol fractions (4.26 *versus* 7.24 mg GAE/g pomace and 4.26 *versus* 9.91 mg GAE/g pomace) and extraction duration of conventional extractions was 360 min, while PLE lasted 15 min and UAE – 20 min. These results could be concluded that innovative extraction methods were more effective than conventional extraction methods for isolation of fractions contained higher TPC. Młynarczyk et al. (2018) reported that elderberry total phenolic content is 364–582 mg GAE/100 g FW or 4917–8974 mg GAE/100 g DW extract [2], Wu et al. (2004) reported that elderberry TPC is 19.5 mg of GAE/g [98], while Lee and Finn (2007) presented that TPC varied from 277 to 582 mg GAE/100 g berries, depending on the growing seasons and cultivators [99]. According to literature data, TPC

may be considered as varied and difficult to compare, because of the differences in elderberry genotypes, analytical methods and sample preparation as well as variable expression of the results. The antioxidant capacity of solid food material can be measure by QUENCHER (**Qu**ick, **E**asy, **N**ew, **Ch**eap, **R**eproducible) approach. The advantage of this procedure is that it does not require extraction or hydrolysis before the measurement of antioxidant capacity. Further, the method does not require to mix the liquid solutions of any compounds to react them and the working hypothesis of the method is to place the solid material and the radical reagent without any extraction steps [79]. TPC of solid residues after non-polar and polar extractions are presented in Tables 24-25, respectively.

Table 24. Total phenolic content of *S. nigra* solid residues after non-polar conventional, high pressure and ultrasound-assisted extractions

Solid residue	Extraction conditions	TPC, mg GAE/g residue	TPC, mg GAE/g pomace*
Conventional extraction methods			
Soxhlet-He	69 °C; 360 min	10.49 ± 0.47^{a}	8.84 ± 0.40^{a}
High pressure and ultrasound-assisted extraction methods			
SFE-CO_2	35 MPa; 53 °C; 105 min	14.51 ± 0.35^{b}	12.47 ± 0.30^{b}
PLE-He-1	10 MPa; 60 °C; 15 min	16.29 ± 1.06^{c}	13.62 ± 0.88^{bc}
PLE-He-2	10 MPa; 60 °C; 30 min	15.01 ± 1.18^{bc}	12.63 ± 1.00^{b}
UAE-He	40 °C; 20 min	17.89 ± 0.67^{d}	14.34 ± 0.53^{c}

*mg GAE/g pomace: TPC, mg GAE/g extract were recalculated into TPC, mg GAE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

Table 25. Total phenolic content of *S. nigra* solid residues after polar conventional, high pressure and ultrasound-assisted extractions

Solid residue	Extraction conditions	TPC, mg GAE/g residue	TPC, mg GAE/g pomace*
Polar extract from starting material Conventional extraction methods			
Soxhlet-He-Ac-EtOH	60 °C; 360 min	10.99 ± 0.22^{c}	10.26 ± 0.20^{c}
SLE-He-Ac-EtOH-H_2O	40 °C; 360 min	14.43 ± 0.53^{d}	13.07 ± 0.48^{d}
Polar extracts from solid residue after SFE-CO_2; High pressure and ultrasound-assisted extraction methods			
PLE-EtOH-2	10 Mpa; 60 °C; 30 min	4.78 ± 0.36^{b}	4.43 ± 0.34^{b}
PLE-EtOH1-H_2O	10 Mpa; 40 °C; 15 min	3.23 ± 0.30^{a}	2.92 ± 0.27^{a}
PLE-H_2O-1	10 Mpa; 40 °C; 15 min	4.26 ± 0.38^{ab}	3.72 ± 0.33^{ab}
PLE-H_2O-2	10 Mpa; 40 °C; 30 min	4.73 ± 0.47^{b}	3.70 ± 0.36^{ab}
UAE-EtOH-H_2O	40 °C; 20 min	14.93 ± 1.06^{d}	13.78 ± 0.98^{d}

*mg GAE/g pomace: TPC, mg GAE/g extract were recalculated into TPC, mg GAE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

TPC of solid residues after PLE and SFE-CO_2 was, on average, 12.9 mg GAE/g pomace without significant differences. The lowest TPC, 8.8 mg GAE/g pomace, had solid residue after

conventional Soxhlet extraction. Based on the data presented in Table 27, the lowest TPC (2.92-3.72 mg GAE/g pomace) had solid residues after PLE with H_2O and the highest TPC, 13.8 mg GAE/g pomace, was determined to solid residues after ultrasound-assisted and solid-liquid extractions.

3.4.2. The $ABTS^{\bullet+}$ scavenging assay

Trolox equivalent antioxidant capacity (TEAC) method or ABTS radical cation decolorization assay uses a diode-array spectrophotometer to measure the loss of colour when an antioxidant is added to the blue–green chromophore $ABTS^{\bullet+}$ (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)). The antioxidant reduces $ABTS^{\bullet+}$ to ABTS and decolorize it [100].

$ABTS^{\bullet+}$ scavenging properties of non-polar and polar extracts are shown in Tables 26-27, respectively. The highest $ABTS^{\bullet+}$ scavenging properties was obtained to non-polar extract after UAE with hexane, 1.2 mg TE/g pomace, and 22% lower result was obtained to extract after PLE with hexane (15 min). It should be noted, that these two non-polar extracts amounted in the highest $ABTS^{\bullet+}$ scavenging properties. Non-polar elderberry extract after PLE with hexane (30 min) had 30% lower $TEAC_{ABTS}$ value than the strongest non-polar extract after UAE (0.86 *versus* 1.22 mg TE/g pomace) and 10% lower $TEAC_{ABTS}$ value than extract after PLE (15 min) (0.86 *versus* 0.95 mg TE/g pomace). It should be noted that SFE-CO_2 extract had 40% lower $TEAC_{ABTS}$ value, but this extraction was hexane-free. Further, the lowest $ABTS^{\bullet+}$ scavenging properties were obtained to extracts after conventional extraction methods, on average 0.28 mg TE/g pomace, and this result was 62-77% lower than $ABTS^{\bullet+}$ scavenging properties of non-polar extracts isolated during innovative extractions (0.28 *versus* 0.73 mg TE/g pomace and 0.28 *versus* 1.22 mg TE/g pomace, respectively).

Table 26. $ABTS^{\bullet+}$ scavenging properties of *S. nigra* non-polar extracts after conventional, high pressure and ultrasound-assisted extractions

Non-polar extract from starting material	**Extraction conditions**	**$TEAC_{ABTS}$, mg TE/g extract**	**$TEAC_{ABTS}$, mg TE/g pomace***
Conventional extraction methods			
Soxhlet-He	69 °C; 360 min	1.85 ± 0.12^{a}	0.29 ± 0.02^{a}
SLE-He	60 °C; 360 min	1.99 ± 0.11^{a}	0.27 ± 0.02^{a}
High pressure and ultrasound-assisted extraction methods			
SFE-CO_2	35 Mpa; 53 °C; 105 min	5.16 ± 0.29^{b}	0.73 ± 0.04^{b}
PLE-He-1	10 Mpa; 60 °C; 15 min	5.80 ± 0.28^{bc}	0.95 ± 0.06^{d}
PLE-He-2	10 Mpa; 60 °C; 30 min	5.43 ± 0.05^{b}	0.86 ± 0.01^{c}
UAE-He	40 °C; 20 min	6.14 ± 0.07^{c}	1.22 ± 0.02^{e}

*mg TE/g pomace: $TEAC_{ABTS}$, mg TE/g extract were recalculated into $TEAC_{ABTS}$, mg TE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

Table 27 presented that $ABTS^{\bullet+}$ scavenging properties of polar elderberry extracts were in the range of 13.06-155.54 mg TE/g extract, while the highest $TEAC_{ABTS}$ values were obtained to polar PLE (30 min) ethanol extract and the lowest – to acetone extract after SLE (360 min). In addition, the lowest $TEAC_{ABTS}$ values (13.06 and 91.08 mg TE/g extract) were determined to the both acetone extracts, that were obtained after Soxhlet and SLE, as compared to the rest of polar extracts. Further, PLE extraction duration (15 or 30 min) had no impact on $ABTS^{\bullet+}$ scavenging properties (155.35 and 155.54 $TEAC_{ABTS}$ mg TE/g extract). On average, $ABTS^{\bullet+}$ scavenging properties of other, Soxhlet, SLE and UAE, ethanol extracts was, on average, 124.8 mg TE/g extract. $TEAC_{ABTS}$ values of water extracts were in the range of 122.02-135.17 mg TE/g extract, while the water extract after SLE amounted in the highest value and water extract after PLE (15 min) – in the lowest. Further, PLE duration was 24-fold shorter than SLE. The $ABTS^{\bullet+}$ scavenging properties of polar elderberry extracts from 1 g of pomace is also presented (Table 27) and in this case, $TEAC_{ABTS}$ values were in rage of 0.26-28.74 mg TE/g pomace, while water fraction after PLE (30 min) provides the highest $TEAC_{ABTS}$ values and SLE acetone fraction – the lowest. $ABTS^{\bullet+}$ scavenging properties of Soxhlet acetone extract was 9.5-fold stronger than SLE acetone extract (2.47 *versus* 0.26 mg TE/g pomace). However, these two acetone extracts were with the lowest $ABTS^{\bullet+}$ scavenging properties, as compared to all polar elderberry extracts. As presented in Table 27, $TEAC_{ABTS}$ values of polar ethanol extracts were in the range of 8.43-16.00 mg TE/g pomace, while the highest $TEAC_{ABTS}$ value was determined to UAE ethanol extract and the lowest – to Soxhlet ethanol extract. In addition, UAE ethanol extract in 18-fold shorter extraction time (360 min *versus* 20 min) amounted in 47% higher $ABTS^{\bullet+}$ scavenging properties (8.43 *versus* 16.00 mg TE/g pomace), as compared to Soxhlet-ethanol extract. $ABTS^{\bullet+}$ scavenging properties of ethanol extracts after conventional extraction methods did not significantly differ and was obtained 8.43-8.74 mg TE/g pomace.

Table 27. ABTS•+ scavenging properties of *S. nigra* polar extracts after conventional, high pressure and ultrasound-assisted extractions

Polar extract	Extraction conditions	$TEAC_{ABTS}$, mg TE/g extract	$TEAC_{ABTS}$, mg TE/g pomace*
Polar extract from starting material Conventional extraction methods			
Soxhlet-He-Ac	50 °C; 360 min	91.08 ± 6.12^{b}	2.47 ± 0.20^{b}
Soxhlet-He-Ac-EtOH	70 °C; 360 min	126.08 ± 4.83^{cde}	8.43 ± 0.40^{c}
SLE-He-Ac	40 °C; 360 min	13.06 ± 0.63^{a}	0.26 ± 0.02^{a}
SLE-He-Ac-EtOH	60 °C; 360 min	124.04 ± 3.12^{cd}	8.74 ± 0.27^{c}
SLE-He-Ac-EtOH-H_2O	40 °C; 360 min	135.17 ± 0.76^{f}	12.77 ± 0.09^{g}
Polar extracts from solid residue after SFE-CO_2 o.c.; High pressure and ultrasound-assisted extraction methods			
PLE-EtOH-1	10 Mpa; 60 °C; 15 min	155.35 ± 3.03^{g}	12.70 ± 0.30^{g}
PLE-EtOH-2	10 Mpa; 60 °C; 30 min	155.54 ± 0.95^{g}	11.53 ± 0.09^{e}
PLE-EtOH-1-H_2O	10 Mpa; 40 °C; 15 min	125.06 ± 2.57^{cde}	12.09 ± 0.30^{f}
PLE-H_2O-1	10 Mpa; 40 °C; 15 min	122.02 ± 0.86^{c}	15.38 ± 0.13^{h}
PLE-H_2O-2	10 Mpa; 40 °C; 30 min	131.51 ± 0.29^{e}	28.74 ± 0.08^{j}
UAE-EtOH	40 °C; 20 min	124.24 ± 2.21^{cd}	16.00 ± 0.35^{i}
UAE-EtOH-H_2O	40 °C; 20 min	130.89 ± 0.48^{def}	10.05 ± 0.05^{d}

*mg TE/g pomace: $TEAC_{ABTS}$, mg TE/g extract were recalculated into $TEAC_{ABTS}$, mg TE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$)

$TEAC_{ABTS}$ values of ethanol extract after innovative extraction methods differs 11.53 (after PLE)-16.00 (after UAE) mg TE/g pomace. Evaluating the efficiency of innovative extraction techniques, PLE duration (15 or 30 min) significantly affected $ABTS^{\bullet+}$ scavenging properties of ethanol extracts and was obtained 11.53 mg TE/g pomace (after 30 min) and 12.70 mg TE/g pomace (after 15 min). This tendency could be explained by thermal degradation of bioactive constituents during longer, 30 min, extraction. $TEAC_{ABTS}$ values of polar hydrophilic extracts were in the range of 10.05-28.74 mg TE/g pomace, while the lowest $ABTS^{\bullet+}$ scavenging properties were determined to UAE water extract and the highest, as mentioned before, to – PLE water extract (30 min). UAE hydrophilic extract amounted in 21.3% lower $TEAC_{ABTS}$ value, as compared to conventional SLE extraction, but UAE was 18-fold shorter than SLE (20 min *versus* 360 min). $ABTS^{\bullet+}$ scavenging properties of UAE hydrophilic extract recovered from other innovative extractions with H_2O in the range of 35-83% (10.0 *versus* 28.7 and 10.0 *versus* 12.09 mg TE/g pomace). Evaluating the efficiency of PLE, PLE duration (15 or 30 min) significantly affected $ABTS^{\bullet+}$ scavenging properties of hydrophilic extracts and the stronger PLE-H_2O extract was after 30 min (15.38 *versus* 28.74 mg TE/g pomace). In the literature data, elderberries $ABTS^{\bullet+}$ scavenging properties varied from 10.7 mmol Trolox/100 g [13] to 19-24 expressed in IC_{50} (mg/mL) [101].

$ABTS^{\bullet+}$ scavenging properties of solid residues after non-polar and polar extractions are presented in Table 28 and 29, respectively. The $TEAC_{ABTS}$ values of solid residues after non-polar

constituents' isolation varied in a range of 60.25-74.61 mg TE/g pomace. The strongest $ABTS^{\bullet+}$ scavenging properties were determined for solid residues after pressurized-liquid extraction with hexane, while the lowest $TEAC_{ABTS}$ value was determined to solid residues after conventional Soxhlet extraction with hexane (60.25 *versus* 74.61 mg TE/g pomace). $ABTS^{\bullet+}$ scavenging properties yielded 5% lower in solid residues after PLE with hexane (30 min) than in solid residues after PLE, which lasted 15 min (71.28 *versus* 74.61 mg TE/g pomace). $TEAC_{ABTS}$ value was 5% lower for solid residues after UAE than after PLE (15 min) (68.13 *versus* 71.28 mg TE/g pomace), followed by the lowest $TEAC_{ABTS}$ values of solid residues after SFE-CO_2 (64.74 mg TE/g pomace), as compared to $TEAC_{ABTS}$ values of solid residues after innovative extraction methods.

Table 28. $ABTS^{\bullet+}$ scavenging properties of *S. nigra* solid residues after non-polar conventional, high pressure and ultrasound-assisted extractions

Solid residue	**Extraction conditions**	**$TEAC_{ABTS}$, mg TE/g residue**	**$TEAC_{ABTS}$, mg TE/g pomace***
Conventional extraction methods			
Soxhlet-He	69 °C; 360 min	71.56 ± 0.36^{a}**	60.25 ± 0.31^{a}
High pressure and ultrasound-assisted extraction methods			
SFE-CO_2	35 Mpa; 53 °C; 105 min	75.32 ± 0.95^{b}	64.74 ± 0.81^{b}
PLE-He-1	10 Mpa; 60 °C; 15 min	89.25 ± 1.15^{d}	74.61 ± 0.96^{e}
PLE-He-2	10 Mpa; 60 °C; 30 min	84.71 ± 0.52^{c}	71.28 ± 0.44^{d}
UAE-He	40 °C; 20 min	85.01 ± 2.64^{c}	68.13 ± 2.11^{c}

*mg TE/g pomace: $TEAC_{ABTS}$, mg TE/g extract were recalculated into $TEAC_{ABTS}$, mg TE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$)

Based on the data presented in Table 29, the lowest $ABTS^{\bullet+}$ scavenging properties had solid residues after PLE with ethanol-water (16.11 mg TE/g pomace), while $ABTS^{\bullet+}$ scavenging properties of solid residues after UAE with ethanol-water were 2-fold higher (31.18 mg TE/g pomace). $TEAC_{ABTS}$ values of solid residues after SLE were up to 4-fold higher than $TEAC_{ABTS}$ values of solid residues after PLE with ethanol-water (69.03 *versus* 16.11 mg TE/g pomace). In addition, it should be discussed that PLE duration was 30 min, while SLE duration was 360 min. So, PLE was more effective in order to isolate higher amount of bioactive compounds.

Table 29. $ABTS^{\bullet+}$ scavenging properties of solid residues after polar conventional, high pressure and ultrasound-assisted extractions

Solid residue	Extraction conditions	$TEAC_{ABTS}$, mg TE/g residue	$TEAC_{ABTS}$, mg TE/g pomace*
Polar extract from starting material Conventional extraction methods			
Soxhlet-He-Ac-EtOH	60 °C; 360 min	45.54 ± 0.52^{d}	42.49 ± 0.48^{e}
SLE-He-Ac-EtOH-H_2O	40 °C; 360 min	76.23 ± 0.48^{e}	69.03 ± 0.44^{f}
Polar extracts from solid residue after SFE-CO_2 o.c.; High pressure and ultrasound-assisted extraction methods			
PLE-EtOH-2	10 Mpa; 60 °C; 30 min	34.34 ± 0.60^{b}	31.80 ± 0.55^{cd}
PLE-EtOH-1-H_2O	10 Mpa; 40 °C; 15 min	17.83 ± 0.25^{a}	16.11 ± 0.23^{a}
PLE-H_2O-1	10 Mpa; 40 °C; 15 min	37.84 ± 0.68^{c}	33.07 ± 0.59^{d}
PLE-H_2O-2	10 Mpa; 40 °C; 30 min	33.56 ± 1.11^{b}	26.22 ± 0.87^{b}
UAE-EtOH-H_2O	40 °C; 20 min	33.78 ± 0.90^{b}	31.18 ± 0.83^{c}

*mg TE/g pomace: $TEAC_{ABTS}$, mg TE/g extract were recalculated into $TEAC_{ABTS}$, mg TE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$)

Further, PLE duration had impact on $ABTS^{\bullet+}$ scavenging properties on solid residues: extraction, which took 30 min resulted in 26.2 mg TE/g pomace, while 15 min extraction resulted in 33 mg TE/g pomace with significant differences. Further, $ABTS^{\bullet+}$ scavenging properties of solid residues after Soxhlet ethanol extraction amounted in 42.5 mg TE/g pomace, while solid residues after PLE with EtOH extraction resulted in 25% lower $TEAC_{ABTS}$ value (31.8 mg TE/g pomace). In addition, innovative extraction methods were more effective and $TEAC_{ABTS}$ values were higher as compared to $TEAC_{ABTS}$ values of solid residues after traditional extraction techniques.

3.4.3. Oxygen radical absorbance capacity (ORAC) assay

ORAC is an innovative new test tube analysis and measures antioxidant inhibition of peroxyl-radical-induced oxidations and reflects classical radical chain-breaking antioxidant activity by H-atom transfer and can be done using fluorescein as target molecule. From this point of view, this method is more relevant to biological systems. The test is performed using Trolox (a water-soluble analogue of Vitamin E) as a standard to determine the Trolox Equivalent (TE). The ORAC value is then calculated from the Trolox Equivalent and expressed as ORAC units or value. The assay is based on generation of free radical using AAPH (2,2-azobis 2-amidopropane dihydrochloride), when the peroxyl radicals generated from thermal decomposition of AAPH and measurement of decrease in fluorescence in the presence of free radical scavengers [100].

ORAC scavenging properties of non-polar and polar *S. nigra* extracts are presented in Table 30 and 31, respectively. $TEAC_{ORAC}$ values of non-polar elderberry extracts were in rage of 27.34-69.43 mg TE/g extract, while the highest $TEAC_{ORAC}$ value was determined to PLE (15 min) hexane

extract and the lowest to – SLE hexane extract. SFE-CO_2, Soxhlet and PLE (30 min) amounted in about 48.55 mg TE/g extract, without significant differences. In addition, it should be discussed that Soxhlet extraction duration was 3.42-18-fold longer than innovative extractions (360 min *versus* 105 and 20 min, respectively).

Table 30. ORAC scavenging properties of non-polar elderberry extracts after conventional, high pressure and ultrasound-assisted extractions, obtained from *S. nigra* pomace

Non-polar extract from starting material	Extraction conditions	$TEAC_{ORAC}$, mg TE/g extract	$TEAC_{ORAC}$, mg TE/g pomace*
Conventional extraction methods			
Soxhlet-He	69 °C; 360 min	47.28 ± 1.96^{b}	7.57 ± 0.22^{b}
SLE-He	60 °C; 360 min	27.34 ± 2.21^{a}	3.68 ± 0.30^{a}
High pressure and ultrasound-assisted extraction methods			
SFE-CO_2	35 MPa; 53 °C; 105 min	48.74 ± 4.46^{b}	6.85 ± 0.63^{b}
PLE-He1	10 MPa; 60 °C; 15 min	69.43 ± 3.01^{d}	11.39 ± 0.45^{c}
PLE-He2	10 MPa; 60 °C; 30 min	49.63 ± 2.69^{b}	7.87 ± 0.41^{b}
UAE-He	40 °C; 20 min	58.41 ± 3.40^{c}	11.60 ± 0.68^{c}

*mg TE/g pomace: $TEAC_{ORAC}$, mg TE/g extract were recalculated into $TEAC_{ORAC}$, mg TE/g pomace, of which dry matter was 94% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$)

ORAC scavenging properties of non-polar elderberry extracts from 1 g of pomace is also presented (Table 30) and in this case, $TEAC_{ORAC}$ values were in rage of 3.68-11.60 mg TE/g pomace, while non-polar fraction after UAE provided the highest $TEAC_{ORAC}$ value and SLE hexane fraction – the lowest. In addition, the PLE extraction time had impact on ORAC scavenging properties of extracts and the stronger extract was obtained after 15 min (11.39 *versus* 7.87 mg TE/g pomace). $TEAC_{ORAC}$ value of SFE-CO_2 was 9.5% lower (6.85 *versus* 7.57 mg TE/g pomace), as compared to $TEAC_{ORAC}$ value of non-polar extract after Soxhlet. Still, SFE CO_2 is environmental friendly method and reduced extraction duration from 360 min to 105 min, as compared to Soxhlet extraction.

As presented in Table 31, $TEAC_{ORAC}$ values of polar elderberry extracts were in rage of 2.13-66.42 mg TE/g pomace, while the highest $TEAC_{ORAC}$ value was determined to PLE (30 min) hydrophilic extract and the lowest – to SLE semi-polar extract. Evaluating the ORAC scavenging properties of hydrophilic extracts, PLE duration (15 or 30 min) significantly affected the scavenging properties of extracts and 15 min PLE resulted in up to 2.7-fold lower values (24.51 *versus* 66.42 mg TE/g pomace). Polar extract after PLE-EtOH-H_2O amounted in 14.7% higher $TEAC_{ORAC}$ value (28.74 *versus* 24.51 mg TE/g pomace), as compared to PLE (15 min)-H_2O extract.

The weakest ORAC scavenging properties (13.17 mg TE/g pomace) were determined to UAE hydrophilic extract, as compared to other hydrophilic extracts. However, $TEAC_{ORAC}$ value of UAE-

EtOH-H_2O extract was 19% lower (13.17 *versus* 16.26 mg TE/g pomace), but extraction duration – 18-fold shorter (360 min *versus* 20 min), as compared to conventional SLE.

Table 31. ORAC scavenging properties of polar extracts after conventional, high pressure and ultrasound-assisted extractions, obtained from *S. nigra* pomace and solid residues

Polar extract	Extraction conditions	$TEAC_{ORAC}$, mg TE/g extract	$TEAC_{ORAC}$, mg TE/g pomace*
Polar extract from starting material Conventional extraction methods			
Soxhlet-He-Ac	50 °C; 360 min	336.21 ± 1.21^{f}	9.11 ± 0.04^{b}
Soxhlet-He-Ac-EtOH	60 °C; 360 min	271.67 ± 9.56^{d}	18.17 ± 0.67^{d}
SLE-He-Ac	40 °C; 360 min	106.61 ± 2.87^{a}	2.13 ± 0.06^{a}
SLE-He-Ac-EtOH	60 °C; 360 min	414.22 ± 1.49^{g}	29.20 ± 0.13^{f}
SLE-He-Ac-EtOH-H_2O	40 °C; 360 min	243.10 ± 4.62^{c}	16.26 ± 0.40^{d}
Polar extracts from solid residue after SFE-CO_2 o.c.; High pressure and ultrasound-assisted extraction methods			
PLE-EtOH-1	10 Mpa; 60 °C; 15 min	103.28 ± 3.51^{a}	8.44 ± 0.29^{b}
PLE-EtOH-2	10 Mpa; 60 °C; 30 min	102.00 ± 6.08^{a}	7.56 ± 0.45^{b}
PLE-EtOH-1-H_2O	10 Mpa; 40 °C; 15 min	227.97 ± 21.84^{c}	28.74 ± 2.90^{f}
PLE-H_2O-1	10 Mpa; 40 °C; 15 min	194.45 ± 3.03^{b}	24.51 ± 0.38^{e}
PLE-H_2O-2	10 Mpa; 40 °C; 30 min	303.89 ± 13.75^{e}	66.42 ± 0.76^{h}
UAE-EtOH	40 °C; 20 min	314.44 ± 11.49^{ef}	40.50 ± 2.39^{g}
UAE-EtOH-H_2O	40 °C; 20 min	171.47 ± 11.54^{b}	13.17 ± 1.09^{c}

*mg TE/g pomace: $TEAC_{ABTS}$, mg TE/g extract were recalculated into $TEAC_{ABTS}$, mg TE/g pomace, of which dry matter was 93.82% (Table 17). Average values of four replicates ± SD. Different superscript letters in the same column indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$)

Among ethanol extracts, the strongest ORAC scavenging properties (40.50 mg TE/g pomace) was determined to UAE ethanol extract. $TEAC_{ORAC}$ values of PLE ethanol extracts were in range of 7.6-8.4 mg TE/g pomace, without significant differences. These, mentioned PLE-EtOH extracts, were up to 2-fold weaker than Soxhlet ethanol extract, but PLE duration was 12-24-fold shorter than Soxhlet (15 and 30 min *versus* 360 min). Results presented in Table 31 could be concluded that the strongest ORAC scavenging properties had hydrophilic extracts (13-66 mg TE/g pomace) and the weakest – acetone extracts (2-9 mg TE/g pomace). The results reported in the literature on the elderberry extracts antioxidant potential may be considered as varied and difficult to compare, because of the differences in elderberry genotypes, growing conditions, analytical methods and sample preparation as well as variable expression of the results. However, Schmitzer et al. (2017) after review of literature data noted that *S. nigra* fruits have higher antioxidant capacity than vitamin C or E and are thus capable of enhancing immune system and using the ORAC method, Wu et al. (2004) showed that especially *S. nigra* berries possess a much higher potential than cranberry and blueberry, two fruits praised for their high antioxidant potential [1, 98].

3.4.4. Development of multi-step extraction schemes for isolation non-polar and polar fractions from S. nigra pomace with the highest in vitro antioxidant capacity

As described in chapter 3.3.3. elderberry pomace non-polar and polar fractions were isolated by using multi-step extractions (conventional and non-conventional) and due to find out the efficiency of various combined extraction methods, total *in vitro* antioxidant activity was determined (Table 32).

The highest total TPC, $TEAC_{ABTS}$ and $TEAC_{ORAC}$ values allowed to obtain multi-step SFE-CO_2 combined with PLE-H_2O extraction (27.88 mg GAE/ g pomace; 29.47 mg TE/g pomace; 73.27 mg TE/g pomace, respectively), while the lowest TPC, $TEAC_{ABTS}$ value were determined to conventional multi-step Soxhlet extraction (8.75 mg GAE/g pomace and 11.19 mg TE/g pomace, respectively) and the lowest $TEAC_{ORAC}$ value had extracts after multi-step SFE-CO_2 + PLE-EtOH extraction (14.41 mg TE/g pomace).

Solid residues after multi-step SFE-CO_2 combined with PLE-EtOH-H_2O and with PLE-H_2O-2 extractions had the lowest TPC (2.92 and 3.70 mg GAE/g pomace, respectively) and it is 75-80% lower than pomace TPC. The same tendency was determined after solid residues $ABTS^{\bullet+}$ scavenging properties measurement and $TEAC_{ABTS}$ values of solid residues after multi-step SFE-CO_2 combined with PLE-H_2O-2 and PLE-EtOH-H_2O extractions were 2.7-4.3-fold (62.5-77.0%) lower than $TEAC_{ABTS}$ values of pomace (26.22 and 16.11 mg TE/g pomace, respectively, *versus* 70.00 mg TE/g pomace). The highest TPC (13.78 and 13.07 mg GAE/g pomace) had solid residues after SFE-CO_2+UAE and SLE multi-step extractions, while the highest $ABTS^{\bullet+}$ scavenging properties (69.03 and 42.48 mg TE/g pomace) were obtained to solid residues after conventional extractions.

Table 32. *In vitro* antioxidant capacity of extracts and solid residues after proposed extraction schemes for isolation non-polar and polar fractions from *S. nigra* pomace powder after juice processing

SCHEME NR. 1: Soxhlet extraction	SCHEME NR. 2: SLE	SCHEME NR. 3: SFE-CO_2+PLE	SCHEME NR. 4: SFE-CO_2+UAE
1. Soxhlet-He: TPC: 4.69 mg GAE/g pomace TEAC$_{ABTS}$: 0.29 mg TE/g pomace TEAC$_{ORAC}$: 7.57 mg TE/g pomace	1. SLE-He: TPC: 2.94 mg GAE/g pomace TEAC$_{ABTS}$: 0.27 mg TE/g pomace TEAC$_{ORAC}$: 3.68 mg TE/g pomace	1. SFE-CO_2: TPC: 2.44 mg GAE/g pomace TEAC$_{ABTS}$: 0.73 mg TE/g pomace TEAC$_{ORAC}$: 6.85 mg TE/g pomace	1. SFE-CO_2: TPC: 2.44 mg GAE/g pomace TEAC$_{ABTS}$: 0.73 mg TE/g pomace TEAC$_{ORAC}$: 6.85 mg TE/g pomace
2. Soxhlet-He-Ac: TPC: 1.13 mg GAE/g pomace TEAC$_{ABTS}$: 2.47 mg TE/g pomace TEAC$_{ORAC}$: 9.11 mg TE/g pomace	2. SLE-He-Ac: TPC: 0.81 mg GAE/g pomace TEAC$_{ABTS}$: 0.26 mg TE/g pomace TEAC$_{ORAC}$: 2.13 mg TE/g pomace	2. PLE-EtOH-1***: TPC: 7.63 mg GAE/g pomace TEAC$_{ABTS}$:12.70 mg TE/g pomace TEAC$_{ORAC}$: 8.44 mg TE/g pomace	2. UAE-EtOH: TPC: 9.91 mg GAE/g pomace TEAC$_{ABTS}$: 16.00 mg TE/g pomace TEAC$_{ORAC}$: 40.50 mg TE/g pomace
3. Soxhlet-He-Ac-EtOH: TPC: 2.93 mg GAE/g pomace TEAC$_{ABTS}$: 8.43 mg TE/g pomace TEAC$_{ORAC}$: 18.17 mg TE/g pomace	3. SLE-He-Ac-EtOH: TPC: 4.26 mg GAE/g pomace TEAC$_{ABTS}$: 8.74 mg TE/g pomace TEAC$_{ORAC}$: 29.20 mg TE/g pomace	3. PLE-EtOH-1-H_2O: TPC: 5.92 mg GAE/g pomace TEAC$_{ABTS}$: 12.09 mg TE/g pomace TEAC$_{ORAC}$: 28.74 mg TE/g pomace	3. UAE-EtOH-H_2O: TPC: 9.19 mg GAE/g pomace TEAC$_{ABTS}$: 10.05 mg TE/g pomace TEAC$_{ORAC}$: 13.17 mg TE/g pomace
SR*** TPC: 10.26 mg GAE/g pomace TEAC$_{ABTS}$: 42.49 mg TE/g pomace	4. SLE-He-Ac-EtOH-H_2O: TPC: 11.00 mg GAE/g pomace TEAC$_{ABTS}$: 12.77 mg TE/g pomace TEAC$_{ORAC}$: 16.26 mg TE/g pomace	SR TPC: 2.92 mg GAE/g pomace TEAC$_{ABTS}$: 16.11 mg TE/g pomace	SR TPC: 13.78 mg GAE/g pomace TEAC$_{ABTS}$: 31.18 mg TE/g pomace
$\sum\tau$*: 1080 min **$\sum$TPC: 8.75 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 11.19 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 34.48 mg TE/g pomace**	SR TPC: 13.07 mg GAE/g pomace TEAC$_{ABTS}$: 69.03 mg TE/g pomace	**$\sum\tau$: 1080 min** **$\sum$TPC: 15.19 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 25.52 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 44.03 mg TE/g pomace**	**$\sum\tau$: 145 min** **$\sum$TPC: 21.54 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 26.78 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 60.52 mg TE/g pomace**
	$\sum\tau$: 1440 min **$\sum$TPC: 19.01 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 22.04 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 51.27 mg TE/g pomace**		

SCHEME NR. 5: SFE-CO_2+PLE-EtOH-1**	SCHEME NR. 6: SFE-CO_2+PLE-EtOH-2**	SCHEME NR. 7: SFE-CO_2+PLE-H2O-1**	SCHEME NR. 8: SFE-CO_2+PLE-H2O-2**
1. SFE-CO_2: TPC: 2.44 mg GAE/g pomace TEAC$_{ABTS}$: 0.73 mg TE/g pomace TEAC$_{ORAC}$: 6.85 mg TE/g pomace	1. SFE-CO_2: TPC: 2.44 mg GAE/g pomace TEAC$_{ABTS}$: 0.73 mg TE/g pomace TEAC$_{ORAC}$: 6.85 mg TE/g pomace	1. SFE-CO_2: TPC: 2.44 mg GAE/g pomace TEAC$_{ABTS}$: 0.73 mg TE/g pomace TEAC$_{ORAC}$: 6.85 mg TE/g pomace	1. SFE-CO_2: TPC: 2.44 mg GAE/g pomace TEAC$_{ABTS}$: 0.73 mg TE/g pomace TEAC$_{ORAC}$: 6.85 mg TE/g pomace
2. PLE-EtOH-1: TPC: 7.63 mg GAE/g pomace TEAC$_{ABTS}$: 12.70 mg TE/g pomace TEAC$_{ORAC}$: 8.44 mg TE/g pomace	2. PLE-EtOH-2: TPC: 7.24 mg GAE/g pomace TEAC$_{ABTS}$: 11.53 mg TE/g pomace TEAC$_{ORAC}$: 7.56 mg TE/g pomace	2. PLE-H_2O-1: TPC: 8.24 mg GAE/g pomace TEAC$_{ABTS}$: 15.38 mg TE/g pomace TEAC$_{ORAC}$: 24.51 mg TE/g pomace	2. PLE-H_2O-2: TPC: 25.44 mg GAE/g pomace TEAC$_{ABTS}$: 28.74 mg TE/g pomace TEAC$_{ORAC}$: 66.42 mg TE/g pomace
SR*** TPC: na**** TEAC$_{ABTS}$: na****	SR TPC: 4.43 mg GAE/g pomace TEAC$_{ABTS}$: 31.80 mg TE/g pomace	SR TPC: 3.72 mg GAE/g pomace TEAC$_{ABTS}$: 33.07 mg TE/g pomace	SR TPC: 3.70 mg GAE/g pomace TEAC$_{ABTS}$: 26.22 mg TE/g pomace
$\sum\tau$: 120 min **$\sum$TPC: 10.70 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 13.43 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 15.29 mg TE/g pomace**	**$\sum\tau$: 145 min** **$\sum$TPC: 9.86 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 12.26 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 14.41 mg TE/g pomace**	**$\sum\tau$: 120 min** **$\sum$TPC: 10.68 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 16.11 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 31.36 mg TE/g pomace**	**$\sum\tau$: 135 min** **$\sum$TPC: 27.88 mg GAE/g pomace** **$\sum$TEAC$_{ABTS}$: 29.47 mg TE/g pomace** **$\sum$TEAC$_{ORAC}$: 73.27 mg TE/g pomace**

*$\sum\tau$: total extraction time duration; **: PLE-EtOH-1 and PLE-H_2O-1 duration 15 min; PLE-EtOH-2 PLE-H_2O-2 duration 30 min; ***SR: *in vitro* antioxidant capacity of solid residues after multi-step extractions; ****na: not applicable, because solid residue after PLE-EtOH-1 was used for PLE-EtOH-1-H_2O extraction.

3.4.5. Measurement of oxidation induction period by Oxipres

Oxidation induction periods of rapeseed oil and rapeseed oil with target non-polar and polar elderberry pomace extracts additives were analysed by accelerated oil stability method (Oxipres) at 110 °C, while the system was filled with oxygen at 0.5 MP [4]. The results are presented in Table 33 and Figure 24.

Table 33. Induction periods of rapeseed oil and rapeseed oil with target elderberry extracts additives

Sample	Elderberry extract additive, %	Induction period (IP) of rapeseed oil (R.o.) + target extract
Rapeseed oil (R.o.)	–	4.06 ± 0.02^{b}
R.o. + Soxhlet-He extract	1	4.20 ± 0.01^{b}
R.o. + Soxhlet-He extract	5	3.56 ± 0.04^{a}
R.o. + SLE-He extract	0.5	4.12 ± 0.06^{b}
R.o. + SLE-EtOH extract	0.5	4.11 ± 0.05^{b}
R.o. + SFE-CO_2 extract	0.5	4.14 ± 0.02^{b}
R.o. + PLE-He-1 extract	0.5	4.87 ± 0.02^{c}
R.o. + UAE-He extract	0.5	3.56 ± 0.08^{a}

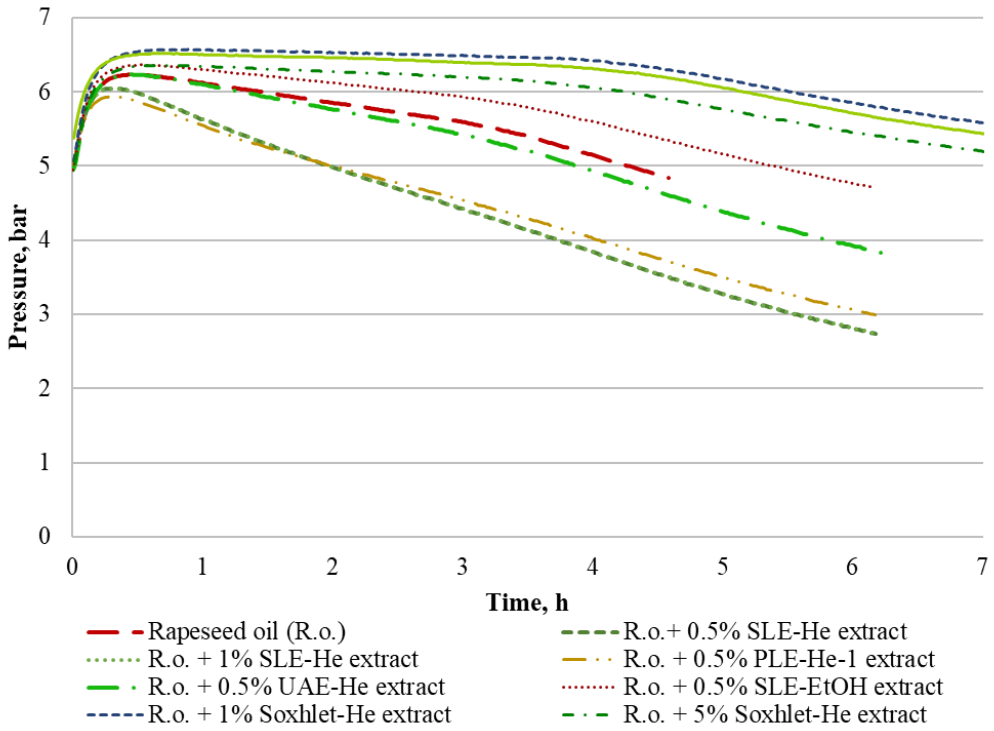


Figure 24. Oxidation curves of rapeseed oil and rapeseed oil with target elderberry pomace extracts (Soxhlet-He; SFE-CO_2; SLE-He; SLE-EtOH; PLE-He-1; UAE-He) additives (0.5%, 1%, 5%) by Oxipres method

The induction periods of rapeseed oil with target elderberry pomace extracts were in the range of 3.56-4.87, while rapeseed oil induction period was 4.06. The longest induction period was in the mixture of rapeseed oil with 0.5% elderberry pomace PLE-He extract additive, while induction period was prolonged 19.95% as compared to rapeseed oil induction period (4.87 *versus* 4.06). The induction period was significantly decreased in the mixture of rapeseed oil with 5% additive of elderberry pomace Soxhlet-He and UAE-He extracts, and induction period was 12.3% lower as compared to induction period of rapeseed oil without additives (3.56 *versus* 4.06). It could be explained by fatty acids composition of elderberry pomace non-polar extracts (chapter 3.5): non-polar elderberry extracts were rich in polyunsaturated fatty acid (~70 %) and these fatty acids should be carefully handled (avoiding contacts with oxygen, protected against direct light and at lower temperatures), because polyunsaturated fatty acids contain two or more double bonds, and these double bonds are prone to oxidation [4]. Aydar et al. (2017) reported that the total phenol content decreased during ultrasound-assisted extraction, while this remark can be explained by the presence of oxygen, which acts as a promoter of non-enzymatic oxidations [102]. This finding could explain why additive of UAE-He extract to rapeseed oil reduced induction period as compared to rapeseed oil without additives.

Further, it should be noted, that 1% additive of Soxhlet-He extract into rapeseed oil, 3.45% prolonged induction period from 4.06 till 4.20, while 0.5% SFE-CO_2, SLE-He and SLE-EtOH extracts prolonged rapeseed oil induction period up to 1.5% (4.12 *versus* 4.06). In addition, elderberry pomace additives from 0.5% till 1% could prolong rapeseed oil induction period, while larger amounts of additives – could reduce.

3.5. Fatty acid composition analysis by gas chromatography (GC-FID)

Healthcare industry is interested in non-traditional plant lipids due to their therapeutic properties and these lipids have become attractive from a nutritional perspective, due to their unique phytochemical composition and antioxidant properties. One of the most important phytochemical characteristics of a lipophilic fraction is fatty acids composition [103–106].

The first step was the isolation of non-polar fraction from *S. nigra* pomace using conventional (Soxhlet, SLE), high-pressure (SFE-CO_2, PLE) or ultrasound extraction method, under different extraction conditions. The second step was the characterization of fatty acids composition of lipophilic extracts by GC-FID. As given in Table 34, the total content of polyunsaturated fatty acid is ~ 70%, while monounsaturated fatty acid content was ~ 13% and the saturated fatty acids have

been found in significantly lower amounts, up to 11%. The dominant fatty acids in all non-polar *S. nigra* pomace extracts were linoleic (40.73-42.35%), α-linolenic (32.04-34.61%), oleic (12.57-13.20%) and palmitic (6.63-9.33%) acids (Figure 25). Some authors presented that linoleic acid and linoleic methyl esters exert antioxidative activity and proposed this as a possible explanation for anticarcinogenic and antiatherogenic effects [107]. Looking at the minor fatty acid profile, myristic (0.04-0.15%), palmitoleic (0.06-0.14%), elaidic (0-0.08%), arachidic (0-0.18), γ-linolenic (0-0.10%) and *cis*-11-eicosenoic (0-0.16%) acids were found only in trace amounts.

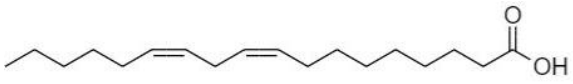
The ratio of saturated/unsaturated (S/U) fatty acids is an important factor in evaluating the nutritive value of lipid content. The biggest S/U ratio was 0.13 in the lipid content extracted by Soxhlet method. This can be explained by the fact that the biggest amount (9.3 %) of saturated palmitic acid was determined in Soxhlet-He non-polar extract. In the lipophilic fractions obtained by another methods S/U ratio was 0.10, which infers that the non-polar extracts were rich in unsaturated fatty acids.

Extraction methods had significant impact on linoleic acid content and the highest amount were extracted during SFE-CO_2, PLE and UAE, without significant differences between the results. The same tendency was noticed with α-linolenic acid content. Palmitic acid content was the highest in Soxhlet-He extract, while in other extracts content was ~6.9% lower. It could be concluded that the conventional extraction methods, such as Soxhlet and SLE, resulted in the highest amount of saturated fatty acids, while non-conventional extraction methods increased not only the content of polyunsaturated fatty acids, but also reduced extraction time up to 24-fold time.

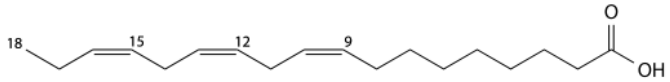
Table 34. The fatty acid content (% of the total GC-FID peak area) of *S. nigra* pomace non-polar extracts, obtained by Soxhlet (360 min; 69 °C), SLE (360 min; 60 °C), PLE (15 and 30 min; 60 °C; 10 MPa), SFE-CO_2 (105 min; 53 °C; 35 MPa) and UAE (20 min; 40 °C) methods

Fatty acid		**Fatty acid content, % of the total GC-FID peak area**					
		Soxhlet-He	SLE-He	SFE-CO_2	PLE-He-1*	PLE-He-2*	UAE-He
Myristic acid	C14:0	0.15 ± 0.02^{c}	0.04 ± 0.00^{a}	0.10 ± 0.00^{b}	0.08 ± 0.01^{ab}	0.11 ± 0.01^{bc}	0.08 ± 0.01^{ab}
Palmitic Acid	C16:0	9.33 ± 0.18^{b}	6.63 ± 0.19^{a}	7.13 ± 0.09^{a}	6.95 ± 0.26^{a}	6.87 ± 0.05^{a}	6.78 ± 0.22^{a}
Palmitoleic Acid	C16:1	0.14 ± 0.01^{b}	0.11 ± 0.00^{ab}	0.07 ± 0.01^{a}	0.06 ± 0.02^{a}	0.09 ± 0.03^{ab}	0.07 ± 0.00^{a}
Stearic Acid	C18:0	1.67 ± 0.01^{a}	1.73 ± 0.06^{a}	1.75 ± 0.00^{a}	1.68 ± 0.02^{a}	1.57 ± 0.12^{a}	1.72 ± 0.01^{c}
Elaidic Acid	C18:1n9t	0.08 ± 0.00	–^{nd**}	–nd	–nd	–nd	–nd
Oleic Acid	C18:1n9C	12.93 ± 0.05^{abc}	12.57 ± 0.05^{a}	12.87 ± 0.04^{abc}	13.20 ± 0.04^{c}	12.68 ± 0.27^{ab}	13.14 ± 0.05^{bc}
Linoleic Acid	C18:2n6C	41.40 ± 0.13^{ab}	40.73 ± 0.00^{a}	42.00 ± 0.05^{bc}	42.40 ± 0.30^{c}	41.47 ± 0.33^{ab}	42.35 ± 0.10^{c}
Arachidic Acid	C20:0	0.11 ± 0.00^{a}	0.18 ± 0.01^{c}	0.16 ± 0.01^{bc}	–nd	–nd	0.12 ± 0.02^{ab}
γ-linolenic Acid	C18:3n6	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.01^{a}	–nd	–nd	0.09 ± 0.01^{a}
cis-11-eicosenoic	C20:0	0.05 ± 0.03^{a}	0.16 ± 0.04^{a}	0.15 ± 0.01^{a}	–nd	–nd	–nd
α-linolenic Acid	**C18:3n3**	32.04 ± 0.06^{a}	32.79 ± 0.20^{ab}	34.13 ± 0.09^{c}	33.99 ± 0.35^{bc}	33.81 ± 0.66^{bc}	34.61 ± 0.04^{c}
∑ Saturated fatty acids		11.26	8.58	9.13	8.71	8.54	8.70
∑ Monounsaturated		13.19	12.84	13.10	13.26	12.77	13.20
∑ Polyunsaturated (ω-6)		73.54	73.62	76.22	76.39	75.27	77.04
Saturated/Unsaturated		0.13	0.10	0.10	0.10	0.10	0.10

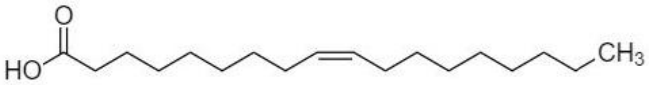
* PLE-He-1: extraction duration 15 min; PLE-He-2: extraction duration 30 min. **nd: not detected. Average values of two replicates ± SD. Different superscript letters in the same row indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$) between fatty acid content (%) obtained by different extraction methods



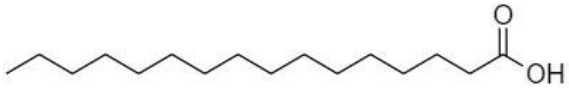
Linoleic acid structure



α-Linolenic acid structure



Oleic acid structure



Palmitic acid structure

Figure 25. Chemical structures of the most abundant fatty acids in various lipophilic extracts from *S. nigra* pomace

Dulf et al. (2013) reported that fatty acid profile of *S. nigra* lipids is dominated by unsaturated fatty acids, such as α-linolenic (41%), linoleic (34%) and oleic acids (14%) [22]. Fazio et al. (2013) studied *S. nigra* seed flour and reported that the most represented fatty acids also were linoleic, oleic and α-linolenic acids (84.0–88.6%). Further, Fazio et al. (2013) also reported that linoleic acid was the main component among all fatty acids (38–49%) and oleic acid content was 13.5% [9]. These results are well agreement with the findings of this researcher work too.

3.6. Volatile compounds of SFE-CO_2 non-polar extract

The odour of *S. nigra* berries and their products is strongly associated to the content of volatile compounds [26]. To the best of our knowledge, previous studies have concentrated mostly on *S. nigra* fruits and flowers volatile compounds determination and the information is not available on elderberry pomace non-polar fraction volatile compounds. Therefore, considerable attention was being paid to determine volatile profile of SFE-CO_2 extract obtained at optimal conditions (35 MPa, 53 °C, 105 min), since this fraction distinguished by pleasant aroma with fresh and green sensorial notes. For this purpose, volatile constituents were isolated by solid phase microextraction (SPME) and identified by gas chromatography-mass spectrometry (GC-MS). The results after SPME-GS-MS are shown in Table 35. Volatile compounds were identified according to their molecular mass and linear retention indexes on DB-5, reported by other authors. Further, there are two types of absolute thresholds: detection (d) and recognition (r). The first being the minimum concentration which can be detected without any requirements to identify or recognize the stimulus, while the second one is the minimum concentration at which a stimulus can be identified or recognized.

Table 35. Volatile compounds profile of *S. nigra* SFE-CO_2 (105 min, 53 °C, 35 MPa) extract

Compound	Formula	Molecular weight	Yield, %	LRI_e*	LRI_l*	Odour threshold**	Ref.
Dimethyl heptane	C_9H_{20}	128	1.266 ± 0.118	805.08	822.2	930; 750-2240	[108]
Methyl tridecane	$C_{14}H_{30}$	198	0.058 ± 0.009	1353.4	1360	42	[109]
Dimethyl undecane	$C_{13}H_{28}$	184	0.112 ± 0.032	1207.8	1213	9.6; 374	[108]
Dimethyl dodecane	$C_{14}H_{30}$	198	0.894 ± 0.135	1247.3	1259	50-11.8	[55]
Benzaldehyde	C_7H_6O	106	0.072 ± 0.002	985.21	965.2	0.01-3400; 0.33-4.1	[108]
Butyl-1-octanol	$C_{12}H_{26}O$	186	0.220 ± 0.019	1296	1277	0.037-0.05; 0.03-5.44	[110]
9-octadecenoamide	$C_{18}H_{35}NO$	281	18.073 ± 1.357	2389.6	2397	nd***	[111]
Methyl octadecane	$C_{19}H_{40}$	268	0.067 ± 0.005	1889.2	1867	0.02; nd	[108]
Methyl nonane	$C_{10}H_{22}$	142	0.032 ± 0.001	955.85	962	nd; 108	[109]
Methyl heptadecane	$C_{18}H_{38}$	254	0.121 ± 0.064	1755.9	1765	nd	[109]
Ethyl cyclohexane	C_8H_{16}	112	0.159 ± 0.022	832.23	826.9	35.6-2700; 120-900	[112]
Dimethyl undecane	$C_{13}H_{28}$	184	0.258 ± 0.044	1217.3	1200.7	9.6; 23-374	[113]
Methylbenzene	C_7H_8	92	0.377 ± 0.069	768.65	770.8	0.6-590; 3.5-260	[108]
Heptadecane	$C_{17}H_{36}$	240	0.786 ± 0.072	1792.9	1765	nd	[109]
Hexadecane	$C_{16}H_{34}$	226	0.508 ± 0.273	1618.9	1600	0.5; nd	[86]
Oleic acid	$C_{18}H_{34}O_2$	282	0.176 ± 0.118	2137.5	2141	nd	[86]
Hexacosane	$C_{26}H_{54}$	366	0.191 ± 0.076	2576.1	2600	nd	[86]
Triacetin	$C_9H_{14}O_6$	218	0.078 ± 0.014	1342	1347	nd	[86]
Dimethyl-benzene	C_8H_{10}	106	0.099 ± 0.077	872.41	863	0.8-2.1; 1.0-3.1	[114]
Dimethyl-octanol (tetrahydrogeraniol)	$C_{10}H_{22}O$	158	0.033 ± 0.005	1077.6	1180.6	0.0-79.0	Nd****
Styrene	C_8H_8	104	0.076 ± 0.011	898.68	895.2	nd	[108]
Linoleic acid	$C_{18}H_{32}O_2$	280	0.160 ± 0.021	2166.5	2132	nd	[86]
Trimethyl-octene	$C_{11}H_{22}$	154	0.039 ± 0.048	1037	1037	2-5; nd	[86]

*LRI_e: experimental linear retention index; LRI_l: linear retention index from literature. **Odour threshold: the first number presents detection (d) and the second – recognition (r) odour threshold. ***nd: not detected; ****nd: from internet source. Average values of two replicates ± SD

From Table 35 appears that the major elderberry pomace non-polar SFE-CO_2 extract volatile compound was oleamide (9-octadecenoamide), which amounted in 18.07% of total volatile compounds peaks area. The second major volatile compound was dimethyl-heptane with yield of 1.27% and the third – was dimethyl-dodecane (0.89%). Volatile compounds such as benzaldehyde, butyl-1-octanol, dimethyl-octanol (tetrahydrogeraniol) were determined in elderberry juice and berries by other researchers [52, 115]. Oleic and linoleic acids also was determined into SFE-CO_2 extract volatile compounds profile and these two fatty acids was determined as the major fatty acids of elderberry non-polar extracts by GC-FID.

Oleamide was determined in *Salvia* spp. SFE-CO_2 extract by Šulniūtė et al. (2017) [111]. Wili et al. (2001) noted that oleamide is the most abundant of the fatty acyl amides in plant and animal

food sources and it was recognised in cocoa powder, bovine, human and goat milks. On the other hand, oleamide is a major primary amide found in mammals and was determined from the cerebral spinal fluid of a sleep deprived cat. Further, oleamide can be produced in brain microsomes from oleic acid and ammonia [116]. Dr. Crozier-Willi from Nestl´e Research Center (Lausanne, Switzerland) (2001) reported that: <…> *I also think that some products on the market may contain oleamide, which has not yet been recognized but is having a psychotropic effect. For example, in the United Kingdom there are drinks that have been traditionally been consumed to help sleep. These are mixed with milk, which may have sedative properties for other reasons. But I would like to see whether these products contain oleamide, which may be contributing to their pacifying effects*. However, oleamide is also known to leach into foods and drinks that are stored in plastic, or plastic-lined, containers, especially those made of polypropylene [117]. In addition, it should be noted that the opinions and findings about oleamide are various and, in our case, we cannot determine from what source it came into extract, from the environment, or this compound naturally occurs in SFE-CO_2 extract. In addition, SFE-CO_2 extract volatile profile mainly depended on compounds, belonging to alkanes, aldehydes and alcohols chemical groups. Benzaldehyde (0.072% of total GC area) is associated with sweet, candy aroma and alcohols butyl octanol (0.220% of total GC area) and dimethyl octanol (0.033% of total GC area) is related with pleasant fruity, citrus odour [57].

3.7. Phytochemical characterization by UPLC-QTOF-MS

3.7.1. Phytochemical characterization of S. nigra pomace extracts in ESI negative mode

Preliminary chemical composition of elderberry pomace extract and target extracts (non-polar Soxhlet-He, SLE-He, SFE-CO_2, PLE-He-1 (15 min), UAE-He; polar Soxhlet-He-Ac, SLE-He-Ac-EtOH, PLE-EtOH-2 (30 min), PLE-H_2O-2 (30 min), PLE-EtOH-1-H_2O, UAE-EtOH, UAE-EtOH-1-H_2O) after conventional, high-pressure and ultrasound-assisted extractions was studied using UPLC-QTOF-MS and all MS data were recorded in ESI negative mode. The results are reported in Table 36. In non-polar extracts 24 compounds was tentatively identified. The peak **1** and **2** gave m/z values of 383.1136 and 179.0561 corresponding to the molecular ion formulas $C_{21}H_{19}O_7$ and $C_6H_{11}O_6$, respectively, and they were identified as hydroxyisoflavonoids and hexose by Metlin. Veberic et al. (2009) reported that one of the major elderberry organic acid is malic acid and the compound **3** gave an m/z value of 133.0141 correlates with the molecular ion formula $C_4H_5O_5$ and was identified as malic acid [25]. The compound **4** with m/z=341.1031 corresponding $C_{19}H_{17}O_6$ ion was identified as polymethoxyflavonoids and it could be caffeic acid hexoside according to Mikulic-

Petkovsek et al. (2015) [10]. The compound **5** with m/z=191.056 corresponding $C_7H_{11}O_6$ was identified as quinic acid by comparison of the retention times with Grunovaite et al. (2016) research [7]. The peak **6** gave m/z values of 111.0088 corresponding to the molecular ion formula $C_5H_3O_3$ and it was identified as furoic acid by Metlin. The compounds **7** and **8** with m/z values of 191.0197 and 153.0193 corresponding $C_6H_7O_7$ and $C_7H_5O_4$, respectively, were identified as citric acid and as dihydroxybenzoic acids by comparison of the retention times with Grunovaite et al. (2016) research [7]. Mikulic-Petkovsek et al. (2015) studied phenolic composition of thirteen elderberry genotypes and their hybrids. After the research were identified 54 phenolic compounds, while quercetin-3-rutinoside and 5-caffeoylquinic acid were the major phenolic compounds. Further, the high diversity of flavonol derivatives were noticed and determined: 13 quercetin glycosides, 7 kaempferol glycosides and 8 isorhamnetin glycosides [118]. According to Mikulic-Petkovsek et al. (2015) determination of elderberry phenolic composition: the compound **9** with m/z=353.0878 corresponding $C_{16}H_{17}O_9$ ion was identified as chlorogenic acid and it could be 3-*O*-Caffeoylquinic acid; the compound **10** with m/z=447.0933 ($C_{21}H_{19}O_{11}$) was identified as flavonol glycoside and it could be 3-*O*-Caffeoylquinic acid; the compound **13** with m/z=367.1035 ($C_{17}H_{19}O_9$) – as hydroxycinnamic acid and it could be 3-feruloylquinic acid; the compound **15** with m/z=609.1461, $C_{27}H_{29}O_{16}$, and the compound **16** with m/z=463.0882 corresponding $C_{21}H_{19}O_{12}$ ion was identified as favonoid glycosides and they could be quercetin 3-*O*-rutinoside and quercetin 3-*O*-galactoside or quercetin 3-*O*-glucoside, respectively, and the compound **17** with m/z=515.1195 ($C_{25}H_{23}O_{12}$) was identified as dicaffeoylquinic acid. Kaack et al. (2008) identified elderberry phenolic compounds in negative mode and reported these compounds: neochlorogenic acid (*m/z* 353) chlorogenic acid (*m/z* 353), cyanidin-3,5-diglucoside (*m/z* 709), cyanidin-3-glucoside (*m/z* 447), quercetin-3-rutinoside (*m/z* 609) and quercetin-3-glucoside (*m/z* 463) [119]. This research is well agreement with Mikulic-Petkovsek et al. (2015) and our work too. The peak **11**, **12, 14** gave m/z values of 465.1038; 579.1355 and 173.0819 and corresponding to the molecular ion formulas of $C_{21}H_{21}O_{12}$; $C_{26}H_{27}O_{15}$ and $C_8H_{13}O_4$ (respectively) and they were identified as flavonoid-*O*-glucuronide, flavonol and dicarboxylic acid by Metlin. The peak **18** was identified with m/z of 193.0506, fitting the molecular ion formula $C_{10}H_9O_4$, it was identified as hydroxycinnamic acids [120]. The peak **19** had an *m/z* of 171.0663, corresponding to the molecular formula $C_8H_{11}O_4$, was identified as unsaturated dicarboxylic acid by Metlin. The peak **20** had an *m/z* of 187.0976, corresponding to the molecular formula $C_9H_{15}O_4$, it was identified as dicarboxylic acids or as Claeys et al. (2012) reported that the major compound eluting with *m/z* 187 was identified as azelaic or nonanedioic acid, a well-known oxidation product of unsaturated fatty acids [121].

Table 36. Elderberry non-polar and polar extracts preliminary chemical composition by UPLC-QTOF-MS in negative ionization mode

Compound**	UPLC-QTOF-MS			Conventional extractions					Innovative extractions							
	RT (min)	MS $[M-H]^-$ m/z	Formula $[M-H]^-$	1*	2*	3*	4*	5*	6*	7*	8*	9*	10*	11*	12*	13*
1. Hydroxyisoflavonoids	0.3-0.5	383.1136	$C_{21}H_{19}O_7$	+	+		+									
2. Hexoze	0.3-0.5	179.0561	$C_6H_{11}O_6$					+			+	+	+		+	+
3. Malic acid	0.3-0.5	133.0142	$C_4H_5O_5$	+		+		+			+	+	+		+	
4. Polymethoxyflavonoids	0.4-0.4	341.1031	$C_{19}H_{17}O_6$		+		+									
5. Quinic acid	0.4-0.5	191.0561	$C_7H_{11}O_6$	+		+		+	+		+	+			+	+
6. Furoic acid	0.6-0.7	111.0088	$C_5H_3O_3$	+		+		+			+	+	+		+	
7. Citric acid	0.7-0.9	191.0197	$C_6H_7O_7$	+		+		+	+			+	+			+
8. Dihydroxybenzoic acids	1.2-1.3	153.0193	$C_7H_5O_4$			+										
9. Chlorogenic acid	1.6-1.8	353.0878	$C_{16}H_{17}O_9$	+					+				+			
10. Flavonol glycoside	1.6-1.8	447.0933	$C_{21}H_{19}O_{11}$	+					+				+			
11. Flavonoid-*O*-glucuronide	1.6-1.8	465.1038	$C_{21}H_{21}O_{12}$	+					+							
12. Flavonol	1.6-1.8	579.1355	$C_{26}H_{27}O_{15}$	+					+				+			
13. Hydroxycinnamic acid	2.0-2.1	367.1035	$C_{17}H_{19}O_9$			+		+								
14. Dicarboxylic acid	2.2-2.3	173.0819	$C_8H_{13}O_4$			+		+		+	+	+	+	+	+	+
15. Favonoid glycoside	2.3-2.3	609.1461	$C_{27}H_{29}O_{16}$	+		+		+	+		+	+	+		+	
16. Flavonoid glycoside	2.4-2.4	463.0882	$C_{21}H_{19}O_{12}$	+				+	+		+					
17. Dicaffeoylquinic acid	2.4-2.5	515.1195	$C_{25}H_{23}O_{12}$			+		+			+					
18. Hydroxycinnamic acid (Ferulic acid)	2.5-2.6	193.0506	$C_{10}H_9O_4$			+		+		+				+	+	+
19. Unsaturated dicarboxylic acid	2.5-2.5	171.0663	$C_8H_{11}O_4$									+	+	+	+	+
20. Dicarboxylic acids (Nonanedioic acid)	2.8-2.8	187.0976	$C_9H_{15}O_4$			+		+	+		+		+	+	+	
21. (S)-Multifidol 2-[apiosyl-(1->6)-glucoside]	2.8-2.8	503.177	$C_{22}H_{31}O_{13}$					+	+				+		+	
22. Dihydroxy fatty acid	5.7-5.8	315.2541	$C_{18}H_{35}O_4$					+		+	+	+	+			+
23. Linolenic Acid	7.5-7.7	277.2173	$C_{18}H_{29}O_2$							+				+		
24. γ-Linoleic acid	8.0-8.2	279.233	$C_{18}H_{31}O_2$							+				+		

*1: pomace; 2: Soxhlet-He; 3: Soxhlet-He-Ac; 4: SLE-He; 5: SLE-He-Ac-EtOH; 6: SFE-CO_2; 7: PLE-He-1; 8: PLE-EtOH-2; 9: PLE-H_2O-2; 10: PLE-EtOH-1-H_2O; 11: UAE-He; 12: UAE-EtOH; 13: UAE-EtOH-1-H_2O. **Confirmed by parent ion mass using free chemical database Metlin.

The peak **21** was identified with *m/z* of 503.177, fitting the molecular ion formula $C_{22}H_{31}O_{13}$, it was identified as (S)-Multifidol 2-[apiosyl-(1->6)-glucoside] by Metlin. This compound belongs to the class of organic compounds known as phenolic glycosides and can be found in fruits [122]. The peaks **23** and **24** had *m/z* of 277.2173 and 279.233, corresponding to the molecular formulas $C_{18}H_{29}O_2$ and $C_{18}H_{31}O_2$, they were identified by linolenic acid and α-linoleic acid. These fatty acids previously were identified in elderberry non-polar extracts by GC-FID (chapter 3.5).

3.7.2. Phytochemical characterization of S. nigra pomace extracts in ESI positive mode

In *S. nigra* berries, juice, pomace and in elderberry non-polar, semi-polar and polar extracts were tentatively identified 24 compounds by UPLC-QTOF-MS in ESI positive mode and preliminary chemical composition is presented in Table 37.

The five major compounds, which were mentioned in other researchers reports, were tentatively identified in elderberry extracts. The compound **8** gave an *m/z* value of 743.2029 that correlates with the molecular ion formula $C_{32}H_{39}O_{20}$ and was identified as cyanidin-3-sambubioside-5-glucoside. The compound **10** gave an *m/z* value of 581.1501 corresponding to the molecular ion formula $C_{26}H_{29}O_{15}$, it was identified as cyanidin-3-sambubioside. Identification of these two compounds was done according to Mandrone et al. (2014) research [101]. According to Wu et al. (2004) research, the compound **11** with *m/z* value of 449.1078 corresponding $C_{21}H_{21}O_{11}$ ion was identified as cyanidin-3-glucoside and the compound **16** with *m/z* value of 611.1607 corresponding $C_{27}H_{31}O_{16}$ ion was identified as cyanidin-3,5-diglucoside, [98]. Veberic et al. (2009) also presented concentrations of the major anthocyanins founded in elderberry and they were: cyanidin-3-sambubioside-5-glucoside ($[M+H]^+$ at *m/z* 743), cyanidin-3,5-diglucoside ($[M+H]^+$ at *m/z* 611), cyanidin-3-sambubioside ($[M+H]^+$ at *m/z* 581) and cyanidin-3-glucoside ($[M+H]^+$ at *m/z* 449). This report is well agreement with Wu et al. research (2004) too. In the group of quercetins, Veberic et al. (2009) determined quercetin-3-glucoside ($[M+H]^+$ at *m/z* 463) and according *m/z* value the compound **21** with $C_{30}H_{39}O_4$ ion was identified as quercetin-3-glucoside. Seabra et al. (2010) reported that cyanidin-3-glucoside is the major anthocyanin in *S. nigra* pomace and the sum of cyanidin-3-glucoside and cyanidin-3-sambubioside represents approximately 90% of the total elderberry anthocyanin content, which ranges from 40 to 150 mg/g DW [8]. The compound **1**, **2**, **3, 7** with m/z 163.0601, 193.0707, 325.1129, 165.0546 with fitting ion formulas in positive mode $C_6H_{11}O$, $C_7H_{13}O_6$, $C_{12}H_{21}O_{10}$, $C_9H_9O_3$, respectively, was identified as organic hydroxy acid, cyclic polyol, fructose dianhydride and hydroxycinnamic acid according to Metlin.

Table 37. Elderberry non-polar and polar extracts preliminary chemical composition by UPLC-QTOF-MS in positive ionization mode

Nr.	Compound*	UPLC-QTOF-MS			Raw material			Conventional extractions ***				Non-conventional extractions ***					
		RT (min)	MS $[M+H]^+$ m/z	Formula $[M+H]^+$	B**	J**	P**	1	2	3	4	5	6	7	8	9	10
1.	Organic hydroxy acid	0.3-0.4	163.0601	$C_6H_{11}O_5$	+						+						
2.	Cyclic polyol	0.3-0.4	193.0707	$C_7H_{13}O_6$	+			+									
3.	Fructose dianhydride	0.3-0.5	325.1129	$C_{12}H_{21}O_{10}$							+						
4.	Dehydroascorbic acid	0.6-0.8	175.0237	$C_6H_7O_6$	+	+								+	+		
5.	Citric acid	0.6-0.8	193.0343	C_6H_9O	+	+	+	+		+		+			+		
6.	Sugar alcohol	0.9-1.0	183.0863	$C_6H_{15}O_6$											+		
7.	Hydroxycinnamic acid	0.9-1.1	165.0546	$C_9H_9O_3$	+		+	+		+		+			+		
8.	Cyanidin-3-sambubioside-5-glucoside	1.4-1.6	743.2029	$C_{32}H_{39}O_{20}$	+	+											
9.	Dihydrogen citrate	1.5-1.6	221.0656	$C_8H_{13}O_7$							+						
10.	Cyanidin-3-sambubioside	1.6-1.9	581.1501	$C_{26}H_{29}O_{15}$	+	+	+	+	+	+		+		+	+		+
11.	Cyanidin-3-glucoside	1.7-1.8	449.1078	$C_{21}H_{21}O_{11}$	+	+	+	+	+	+		+		+	+		+
12.	Trimethoxyflavan	2.0-2.1	317.1384	$C_{18}H_{21}O_5$	+												
13.	Flavonoid glucoside	2.0-2.1	757.2186	$C_{33}H_{41}O_{20}$	+	+											
14.	Anthocyanidin-3-*O*-glycosides	2.2-2.3	325.2221	$C_{15}H_{33}O_7$											+		
15.	Quercetin-3-glucoside	2.2-2.4	465.1028	$C_{21}H_{21}O_{12}$	+	+	+	+	+	+	+	+		+	+		
16.	Cyanidin-3,5-diglucoside	2.2-2.4	611.1607	$C_{27}H_{31}O_{16}$	+	+	+	+	+	+	+	+		+	+		+
17.	Delphinidin	2.2-2.4	303.0499	$C_{15}H_{11}O_7$	+		+	+	+	+	+	+		+	+		
18.	Flavone	4.4-4.5	403.1387	$C_{21}H_{23}O_8$					+		+			+			
19.	Trihydroxyflavanone	4.9-5.0	439.2115	$C_{26}H_{31}O_6$									+				
20.	Pentacyclic triterpenoid	6.0-6.1	455.3520	$C_{30}H_{47}O_3$				+	+		+						
21.	Quercetin 3-*O*-glucoside	6.7-6.8	463.2843	$C_{30}H_{39}O_4$	+									+	+		
22.	Phenyl acids	6.7-6.8	165.0910	$C_{10}H_{13}O_2$							+				+	+	+
23.	Limonene	6.7-6.8	151.1117	$C_{10}H_{15}O$										+			
24.	Ergosterol (Vitamin D)	7.6-7.7	411.3621	$C_{29}H_{47}O$									+				

*Confirmed by parent ion mass using free chemical database Metlin.**B: berries; J:juice; P:pomace; ***Elderberry extracts: 1:Soxhlet-He; 2: Soxhlet-He-Ac; 3: SLE-He; 4: SLE-He-Ac-EtOH; 5: SFE-CO_2; 6: PLE-He-1; 7: PLE-EtOH-2; 8: PLE-EtOH-1-H_2O; 9: UAE-He; 10: UAE-EtOH.

The compound **4** ($[M+H]^+$ at *m/z* 175.0237) was determined as dehydroascorbic acid by Hounsome et al. (2009) research [123]. The compounds **5** *m/z* value of 193.0343 corresponding C_6H_9O was identified as citric acid by Zhang et al. (2010) report [124]. The compounds **6** and **9** with *m/z* values of 183.0863 and 221.0656 corresponding $C_6H_{15}O_6$ and $C_8H_{13}O_7$ ion formulas was identified sugar alcohol and dihydrogen citrate by Metlin. The compound **15** ($[M+H]^+$ at *m/z* 465.1028) with fitting ion molecular formula $C_{21}H_{21}O_{12}$ was named quercetin-3-glucoside by Fazio et al. (2013) report [9], while according Nakajima et al. (2004) research the compound **17** with *m/z* value of 303.0499 and fitting $C_{15}H_{11}O_7$ ion formula was described as delphinidin [125]. The compound **18** ($[M+H]^+$ at *m/z* 403.1387) corresponding $C_{21}H_{23}O_8$ ion formula in positive mode was identified as flavone [126], which is flavonoid occurred in fruits peel and according Nakajima et al. (2007), this compound could improve memory impairments [127]. The compound **19** with m/z= 439.2115 corresponding $C_{26}H_{31}O_6$ ion formula was identified as trihydroxyflavanone acid by comparison of the *m/z* values with Metlin and Zhang et al. report (2008). The compound **20** ($[M+H]^+$ at *m/z* 455.3520) corresponding $C_{30}H_{47}O_3$ ion formula in positive mode was identified as pentacyclic triterpenoid [128], the compound **23** ($[M+H]^+$ at *m/z* 151.1117) corresponding $C_{10}H_{15}O$ ion formula was recognized as limonene, according to Kern et al. (2014) report [129] and the last compound **24** ($[M+H]^+$ at *m/z* 411.3621) corresponding $C_{29}H_{47}O$ ion formula in positive mode was identified as ergosterol or vitamin D by comparison of the retention times with Ishii et al. (2009) research [130].

3.8. Quantitative analysis of cyanogenic glycosides constituents

S. nigra berries contain several cyanogenic glycosides, among which sambunigrin is the major constituent. Sambunigrin upon hydrolysis in the gastrointestinal tract is converted to toxic hydrogen cyanide. The potential toxicity of cyanogenic glycoside depends on its capacity to produce HCN, further 1 g of the sambunigrin on hydrolysis can release 91.5 mg HCN (equivalent to 88.1 mg CN^-). In the Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on hydrocyanic acid in flavourings and other food ingredients with flavouring properties (2004) it is reported that the acute lethal oral dose of HCN for humans is 0.5-3.5 mg HCN/kg BW (0.48-3.37 mg CN^-/kg BW) and the clinical signs of intoxication are headache, dizziness, mental confusion, stupor, cyanosis with twitching and convulsions, followed by terminal coma [51]. Therefore, it is very important to consider the safety of various elderberry products. For these reasons, the amount of sambunigrin was analysed in *S. nigra* berries, juice,

pomace after juice processing, solid residue after SFE-CO_2 and selected non-polar and polar fractions after applying different extraction techniques. The results are reported in Tables 38-39.

Table 38. Amount of sambunigrin in *S. nigra* berries, pomace, juice and solid residue after SFE-CO_2 (35 MPa, 53 °C, 105 min)

Sample	Amount of sambunigrin, pg/g of sample	Amount of sambunigrin, pg/100 g berries DW*
Berries	0.90 ± 0.02^{b}	89.45 ± 1.63^{c}
Juice	5.79 ± 0.01^{c}	235.70 ± 0.37^{d}
Pomace	0.66 ± 0.01^{a}	36.53 ± 0.28^{a}
Solid residue after SFE-CO_2	0.89 ± 0.04^{b}	42.70 ± 1.73^{b}

*DW: dry weight (calculations are given in chapter 3.1). Average values of two replicates ± SD. Different superscript letters indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

The lowest cyanogenic glycoside sambunigrin amount (36.53 pg/g 100 g DW of berries) was detected into pomace, while the highest amount (235.70 pg/g 100 g DW of berries) – in elderberry juice. The amount of sambunigrin in pomace after juice processing reduced up to 2.4-fold (89.45 *versus* 36.53 pg/g 100 g berries DW) and up to 2.09-fold in solid residues after SFE-CO_2 (89.45 *versus* 42.70 pg/g 100 g berries DW), as compared to sambunigrin amount in berries. Further, the sambunigrin amount differences among berries and juice (89.45 *versus* 235.70 pg/g 100 g berries DW, respectively) could be explained by berries drying 40 °C for 72 hours, while juice was only freeze-dried. The drying of berries could lead in the degradation of sambunigrin and evaporation of HCN.

Table 39 illustrated that the lowest amount of sambunigrin, 2.80 pg/g 100 g DW of pomace, was determined into polar Soxhlet-Ac extract and the highest amount of sambunigrin, 73.39 pg/g 100 g DW of pomace – into polar PLE-H_2O-1 (15 min) extract. On average, the amount of sambunigrin in polar SLE, PLE, UAE ethanol extracts was 10.28 pg/g 100 g DW of pomace, without significant differences. The amount of sambunigrin differs 0-57.750 pg/g DW 100 g of pomace in polar hydroethanolic PLE and UAE extracts. Partially these differences could be explained by diverse extractions parameters and sample preparation parameters. For example, solid residue after PLE with EtOH (15 min) prior to other step of extraction with H_2O was dried 24 hours at 40 °C temperature. On the other hand, following the UAE extraction protocol (2.5.3.), H_2O portion was straightaway added on solid residue after UAE with EtOH (20 min) without drying. Also, in PLE solid residues were twice affected by 10 MPa pressure and extraction temperatures of 60 °C during PLE-EtOH and 140 °C during PLE-EtOH-H_2O, which lead in the degradation of

sambunigrin and evaporation of HCN, thus suggesting the reason why sambunigrin was not detected in latter sample.

Table 39. Amount of sambunigrin in non-polar and polar *S. nigra* pomace extracts, obtained after conventional (Soxhlet, SLE), high pressure (SFE-CO_2, PLE) and ultrasound-assisted extractions

Extract	Amount of sambunigrin, pg/g extract	Amount of sambunigrin, pg/100 g pomace DW*	Amount of sambunigrin, pg/100 g berries DW
Non-polar extract; conventional extraction methods			
Soxhlet-He	0.69 ± 0.00^{abc}	10.92 ± 0.02^{bc}	6.08 ± 0.01^{b}
SLE-He	0.50 ± 0.00^{a}	6.73 ± 0.01^{ab}	3.75 ± 0.01^{ab}
Non-polar extract; high pressure and ultrasound-assisted extraction methods			
SFE-CO_2	0.67 ± 0.01^{ab}	9.41 ± 0.16^{abc}	5.24 ± 0.09^{b}
PLE-He	0.72 ± 0.00^{abc}	11.84 ± 0.08^{bc}	6.59 ± 0.05^{b}
UAE-He	0.53 ± 0.00^{ab}	10.43 ± 0.17^{bc}	5.81 ± 0.09^{b}
Polar extract from starting material; conventional extraction methods			
Soxhlet-He-Ac	1.03 ± 0.01^{bcd}	2.80 ± 0.03^{a}	1.56 ± 0.02^{a}
SLE-He-Ac-EtOH	1.29 ± 0.04^{d}	9.10 ± 0.31^{abc}	5.07 ± 0.17^{ab}
Polar extract from solid residue after SFE-CO_2; high pressure and ultrasound-assisted extraction methods			
PLE-EtOH	1.20 ± 0.00^{cd}	9.83 ± 0.02^{bc}	5.48 ± 0.01^{b}
PLE-EtOH-1-H_2O	nd**	nd	nd
PLE-H_2O	5.82 ± 0.43^{e}	73.39 ± 5.46^{e}	40.88 ± 3.04^{d}
UAE-EtOH	0.93 ± 0.00^{abc}	11.91 ± 0.09^{c}	6.64 ± 0.05^{b}
UAE-EtOH-H_2O	7.52 ± 0.05^{f}	57.75 ± 0.4^{d}	32.17 ± 0.23^{c}

*DW: dry weight (calculations are given in chapter 3.1). **nd: not detected. Average values of two replicates ± SD. Different superscript letters indicate significant differences (one-way ANOVA and Tukey`s test $p < 0.05$).

The amount of sambunigrin in non-polar elderberry extracts was in the range of 6.73-11.84 pg/g 100 g pomace DW, while the lowest amount was determined into SLE-He extract and the highest – into PLE-He extract, however, without significant differences. Further, amount of sambunigrin was recalculated from pg/g extract into pg/g 100 g berries DW and sambunigrin amounted into range of 1.56-40.88 pg/g 100g berries DW: the lowest amount was determined in Soxhlet-Ac extract, while the highest – in PLE-H_2O extract. Amount of sambunigrin amounted in 3.75-6.59 pg/g 100 g berries DW in non-polar extracts, without significant differences, and in 5.07-6.64 pg/g 100 g berries DW in elderberry ethanolic extracts. It should be noted that PLE-EtOH-1-H_2O and UAE-EtOH-H_2O had the highest amount of sambunigrin (40.88 and 32.17 pg/g 100 g berries DW, respectively).

In the Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on hydrocyanic acid in flavourings and other food ingredients with flavouring properties (2004) it is reported that the acute lethal oral dose of HCN for humans is

0.5-3.5 mg HCN/kg BW (0.48-3.37 mg CN^-/kg BW) and the clinical signs of intoxication are headache, dizziness, mental confusion, stupor, cyanosis with twitching and convulsions, followed by terminal coma [51]. Therefore, it is very important to consider the safety of various elderberry products. In addition, Senica et al. (2016) concluded that type of extraction, time and temperature affected cyanogenic glycosides in various elderberry products, such as juice, liquor, tea. Senica et al. (2016) obtained that the highest amount of sambunigrin was in the juice, about 19 mg/kg, so it can be calculated into 1.74 mg/kg of HCN. However, as mentioned before, for humans' acute lethal oral dose is 0.5-3.5 HCN mg/kg BW, so the person who weighs, for example 70 kg, can get 35-245 mg or 0.38-2.68 g pure sambunigrin. In our research, the amount of sambunigrin was obtained only in trace amounts – 1.56×10^{-12}-235.70×10^{-12} g/g 100 g berries DW (amount of sambunigrin in Soxhlet-Ac extract and elderberry juice, respectively) and human should consume about 17186108-24551660 tons of Soxhlet-Ac extract and 1134574-162688 tons of elderberry juice to get acute lethal dose of HCN. The amount of sambunigrin differs between Senica et al. (2016) and our results and it could be explained by the Veberic et al. (2016) findings, who reported that sambunigrin content could be very individual of every plant and depends on the elderberry growing conditions, while the elderberry synthesis of cyanogenic glycosides is the defence system against herbivores [35, 131].

CONCLUSIONS

1. Elderberry pomace after juice processing amounted 31% of total berries fresh weight, with 15.80 g/100 g of lipid content, 13.00 g/100 g of total nitrogen content and 4.10 g/100 g of mineral content. Pomace moisture content after drying at 40 °C for 72 h was 6.20 g/100 g of sample. Pomace TPC (8.19 mg GAE/g DW) amounted in 23.06% of berries TPC, while juice TPC amounted in 70.58% of berries TPC. Pomace $ABTS^{\bullet+}$ scavenging properties (37.33 mg TE/g DW) yielded in 37.99%, while juice yielded in 61.37% of berries $TEAC_{ABTS}$ value.
2. Optimal supercritical carbon dioxide extraction parameters after CCD-RSM optimization for lipophilic constituent isolation from *S. nigra* pomace was 35 MPa, 53 °C and 105 min. It was determined that 14.05 g/100 g extraction yield was obtained at these optimal conditions. The SFE-CO_2 optimization model was significant (*F*-value 191.18) and the pressure was the most important independent variable (*F*-value 1035.01), followed by extraction temperature (*F*-value 25.59) and time (*F*-value 5.59) variables.
3. Elderberry non-polar fraction amount varied from 13.47 to 19.86 g/100 g pomace, while the highest yield was isolated after UAE and the lowest – after SLE. Environmental-friendly SFE-CO_2 in 3.4-fold shorter extraction time amounted 89% and 104% of the total Soxhlet and SLE yields. Polar constituents amounted 2.00-21.85 g/100 g pomace, while PLE-H_2O was the most effective extraction method and SLE-Ac yielded in the lowest polar fraction amount. Proposed multi-step pomace valorisation (biorefining) scheme of elderberry pomace consecutively combines SFE-CO_2 and PLE-H_2O extractions. This procedure allows to obtain 39.90 g/100 g of extractable constituents (39% non-polar; 61% polar) during 135 min, while extraction duration could be shortened till 60 min. SFE-CO_2 kinetics results determined that 98% of total extraction yield was obtained after the first 30 min. Also, this multi-step extraction scheme offers an advantage of using food-grade solvents CO_2 and H_2O and requires solvent removal only after the second extraction step.
4. Total phenolic content and in vitro radical scavenging capacity for various elderberry extracts ranged from 0.81 to 25.44 mg GAE/g of pomace and from 0.26 to 66.42 mg TE/g of pomace. The highest activity was determined to PLE-H_2O-2 (30 min) extract, while the lowest – for SLE-He-Ac extract. Proposed biorefining scheme of elderberry pomace (SFE-CO_2+ PLE-H_2O extractions) allowed to obtain the highest total phenolic content (27.88 mg GAE/g pomace) and the strongest $ABTS^{\bullet+}$ and ORAC scavenging properties (29.47 mg TE/g

pomace and 73.27 mg TE/g pomace, respectively). Solid residues after the last step extraction had 75% lower TPC and 63% lower $TEAC_{ABTS}$ value as compared to unextracted pomace, additionally showing the efficiency of the proposed pomace valorisation scheme (SFE-CO_2 + PLE-H_2O) to isolate valuable constituents with *in vitro* antioxidant properties.

5. The fatty acid of non-polar elderberry extracts showed the presents of 8.54-11.26% saturated fatty acids; 12.77-13.26% monounsaturated fatty acids and 73.54-77.04% polyunsaturated fatty acids. The dominant fatty acids in all non-polar *S. nigra* pomace extracts were linoleic (40.73-42.40%), γ-linolenic (32.04-34.61%), oleic (12.68-13.20%) and palmitic (6.63-9.33%) acids. The highest amounts of these polyunsaturated fatty acids were found in lipophilic fraction after SFE-CO_2 and UAE.
6. The major elderberry pomace non-polar SFE-CO_2 extract volatile compound was oleamide (9-octadecenoamide), which amounted in 18.07% of the total GC peaks area. SFE-CO_2 extract volatile profile mainly depended on compounds, belonging to alkanes, aldehydes and alcohols chemical groups. Benzaldehyde (0.072% of total GC area) is associated with sweet, candy aroma and alcohols butyl octanol (0.220% of total GC area) and dimethyl octanol (0.033% of total GC area) is related with pleasant fruity, citrus odour.
7. In non-polar extracts, 24 compounds were tentatively identified, mainly belonging to chemical groups of flavonoids (favonoid glycoside, hydrocinnamic acid) and organic acids (malic, citric, furoic). In semi-polar and polar extracts, 24 compounds were tentatively identified, mainly belonging to anthocyanins (cyanidin-3-*O*-sambubioside, cyanidin-3-*O*-glucoside, cyanidin-3,5-*O*-diglucoside) and flavonols (quercetin-3-*O*-rutinoside).
8. The amount of major cyanogenic glycoside sambunigrin in target elderberry extracts was obtained only in trace amounts. The highest amount of sambunigrin was determined in elderberry juice (235.70 pg/g 100 g berries DW). In various non-polar and polar elderberry pomace extracts, 1.56-48.8 pg/100 g of sambunigrin was detected, with the highest amount in PLE-H_2O extract and the lowest – in Soxhlet-Ac extract. Therefore, in terms of cyanogenic glycoside content, all obtained elderberry pomace extracts could be regarded as safe, since in order to get acute lethal dose of major toxic metabolite (hydrogen cyanide) human should consume up to 25×10^6 tons of Soxhlet-Ac extract and up to 16×10^4 tons of elderberry juice per day.

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