Chemia Naissensis, Vol 2, Issue 2, REVIEW, 1-25

Sambucus nigra and *Sambucus racemosa* fruit: a schematic review on chemical characterization

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ABSTRACT

Elderberry is a plant which parts are used for healing purposes. It is rich in polyphenolic compounds (anthocyanins, flavonols, phenolic acids, proanthocyanidins). Black elderberry is the most characterized of all elderberry types. In this paper, the emphasis is on published results about the fruit of black elderberry (*Sambucus nigra*) and red elderberry (*Sambucus racemosa*), as well as different cultivars within these species. The first step in chemical analysis of a plant material is the extraction. It is important to choose the appropriate extraction technique and solvent(s) for the extraction. Spectrophotometric methods enable the determination of total phenol content, total monomeric anthocyanin content, antioxidant activity (ABTS $^{\cdot+}$, DPPH$^{\cdot}$, TEAC, β-carotene / linoleic acid assays). High performance liquid chromatography technique combined with appropriate detectors (for carbohydrates and organic acids: HPLC-PDA; for individual phenolic compounds: HPLC-DAD-MS, HPLC-DAD-ESI-MS-MS; for individual anthocyanins: HPLC-DAD–, HPLC-MS-MS, HPLC-UV-MS-MS, HPLC-DAD-ESI-MS, HPLC-DAD-ESI-MS-MS; for proanthocyanins: HPLC-ESI-MS-MS) provides the results about chemical composition, which were determined. Differences in chemical composition are evident between black and red elderberry, and less within different cultivars of the same species. Values for the total anthocyanin content obtained by using the HPLC method are two or more times higher than those obtained spectrophotometrically. The same can be said for the results for phenolic compounds. Elderberry fruit should be more commercialized since the chemical composition makes it a source of a cosmetically active substances.

Keywords: elderberry, chemical composition, Sambucus nigra, Sambucus racemosa

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Introduction

The history of using herbs for healing purposes is as old as humanity. There are many written documents which confirm this statement. Egyptian papyrus (Vinatoru, 2001), recipes written by Hippocrates, Paracelsus, Dioscorides and others showed the use of plants for medicinal purposes (Paulsen, 2010). One of the oldest and very often used species for medical purpose is elderberry. Since the prehistoric times, all parts of elderberry (root, herb, cortex, leaves, flowers, berries) were used as a healing plant material (Akbulut et al., 2009). In that time, the mankind didn't know about bioactive molecules.

Black elderberry (*Sambucus nigra* L.) and red elderberry (*Sambucus racemosa* L.) belong to the Adoxaceae family (Christensen et al., 2008). Both of these species grow on sunlight-exposed locations. It is interesting that the red elderberry (*S. racemosa*) is the most common and reliable shrub indicator of O_3 phytotoxic effects (Manning, 2005). By using the chemical analyses and appropriate method, it is possible to identify and quantify bioactive compounds. Elderberries are rich in phytochemicals (Thole et al., 2006). Both of mentioned elderberry fruits contain phenolic compounds. Phenolic compounds belong to the class of the bioactive compounds (Mikulic-Petkovsek et al., 2015). Black elderberries contain organic acids, flavonols glycosides (Veberic et al., 2009) and anthocyanins (Wu et al., 2004).

Extraction is a very important step in analyzing of plant species. Before starting an extraction, there are lot of choices to be made – about extraction technique, solvent(s), temperature, extraction time and other extraction parameters. Bioactive compounds can be identified and characterized from various plant parts such as leaves, stem, flower and fruits (Hernandez et al., 2009). For the identification and quantification of chemical compounds spectrophotometric and HPLC methods are the options. The HPLC analyses provide a simultaneous determination of each chemical compound (Zhang et al., 2010).

There are numerous scientific papers about *Sambucus* species. They all contribute to better chemical characterization and understanding of use for healing purposes. *S. nigra* was investigated by scientists more than other *Sambucus* species. The aim of this work was *de facto* to give in one place as much as possible information about chemical composition and characteristics of *S. nigra* and *S. racemosa* fruit, and also different cultivars of these two species, to make easier a future research by scientists interested in this plant material.

Experimental

A guide for the analysis of *S. nigra* and *S. racemosa* fruit is shown in Figure 1. It is formed on the bases of the published results of authors who studied mentioned plant material. The scheme shows an integrated approach for elderberry study. It starts with a literature review. All sequences of the schematic representation will be discussed in more details further in this paper.

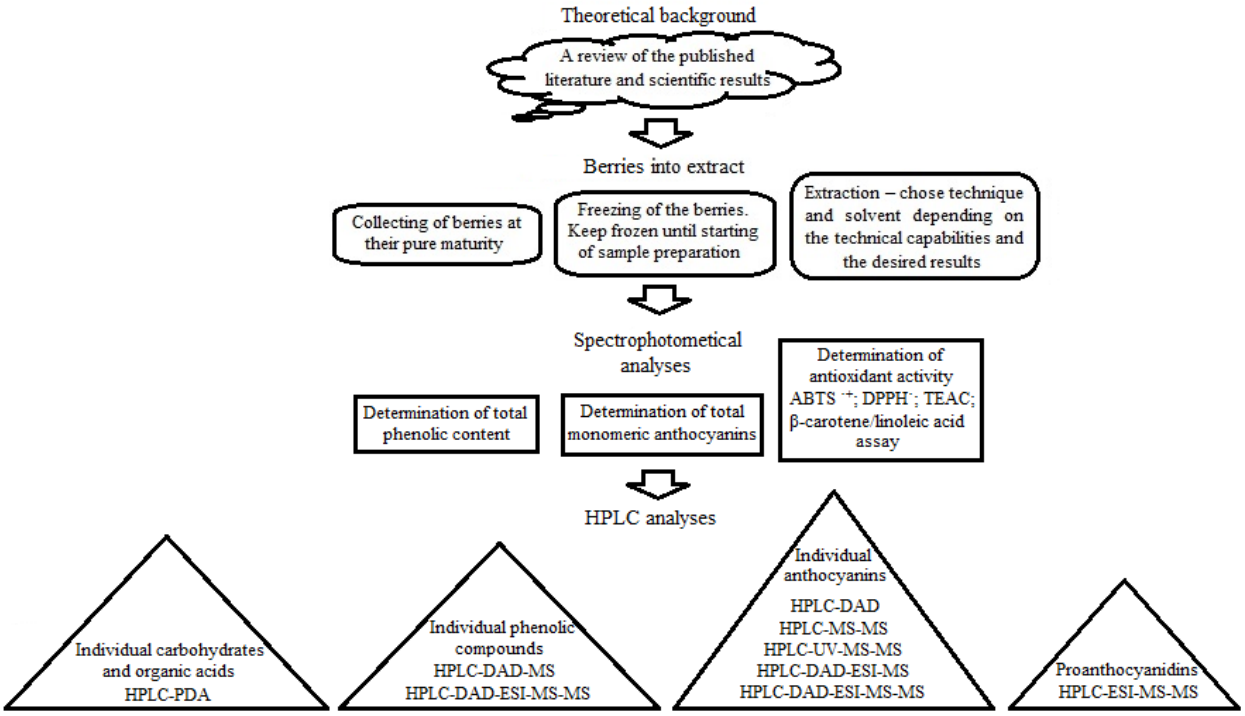

Figure 1. The flow chart of *Sambucus* fruit study

1. Theoretical background

Firstly, published results for *Sambucus* fruits should be studied. Based on the numbers of published papers, *S. racemosa* fruit was less interesting for scientists than *S. nigra*. Generally, there are results of several studies which can characterize *S. nigra* plant material well, but there is not enough published papers on the characterization of *S. racemosa*. In this mini-review paper, several papers with enviable citation were considered and their data for *S. nigra* and *S. racemosa* fruits for different cultivars within the same species are processed.

2. Plant material

The fruits of two *Sambucus* species – *Sambucus nigra* and *Sambucus racemosa* are the focus of this paper. These two species have different genotypes, and the difference between them will be mentioned through the results obtained. All berries have been collected at their full maturity. After that, they were air-dried to constant weight (Dawidowicz et al., 2006; Duymus et al., 2014), frozen at -20 °C (Lee and Finn, 2007; Wu et al., 2004) or -80 °C (Mikulic-Petkovsek et al., 2015). Wu et al. (2004) grinded frozen berries into powder and kept it at -70 °C. Until the beginning of analyses of plant material, it was kept in plastic bags under mentioned conditions.

3. Extraction

The selection of proper extraction method is the basis for further qualitative and quantitative studies of bioactive compounds from plant material (Sasidharan et al., 2011; Smith, 2003). Extraction is the first step of any medicinal plant study; it plays a significant and crucial role on the final result. Extraction methods are sometimes referred as ‘‘sample preparation techniques’’. In the study conducted by Majors (1999), it was confirmed the importance of the sample preparation for analysis.

In order to extract chemical components of interest from the plant material, extraction may play a crucial role in further analyses. Specific nature of the targeted bioactive compounds dictates the selection of solvent system for the extraction. There are many different options for the selection of solvent systems for the extraction from plant material. It is possible to use a single solvent for the extraction, or a mixture. The extraction of hydrophilic compounds is performed using polar solvents such as methanol, ethanol or ethyl-acetate. Target compounds may be non-polar to polar and thermally labile, so the suitability of the methods of extraction must be considered (Sisidharan et al., 2011). The polarity of the targeted compound is the most important factor for the solvent selection. Molecular affinity between the solvent and the solute, mass transfer, use of cosolvent, environmental safety, human toxicity and financial feasibility should also be considered during the selection of the solvent for bioactive compound extraction (Azmir et al., 2013). Since plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals which have different polarity, the question about their identification, separation and characterization needs to be answered. Extraction efficiency of any conventional method mainly depends on the selection of solvents (Cowan, 1999).

Various methods, such as sonification, heating under reflux, Soxhlet extraction are commonly used (Pharmacopoeia of the People’s Republic of China, 2000; The Japanese Pharmacopeia, 2001; United States Pharmacopeia and National Formulary, 2002) for the plant samples extraction. All these techniques have some common objectives: (a) to extract targeted bioactive compounds from complex plant sample, (b) to increase selectivity of analytical methods (Smith, 2003). Some of these techniques are considered as ‘‘green techniques’’ as they comply with the standards set by Environmental Protection Agency, USA (https://www.epa.gov/greenchemistry/basics-green-chemistry#definition). These techniques include less hazardous chemical synthesis, designing safer chemicals and safe solvents auxiliaries, design for energy efficiency, use of renewable feedstock, reducing of derivatives, catalysis, design to prevent degradation, atom economy, and time analysis for pollution prevention and inherently safer chemistry for the prevention of accidents (Azmir et al., 2013). Deep eutectic solvents are considered as an ideal substitute for conventional solvents and they are used for the extraction of plant material (Paiva et al., 2014).

There are conventional methods and numerous other methods established for the extraction of bioactive compounds from plant material. However, there is no standard method. Factors, such as understanding of the nature of plant matrix and chemistry of bioactive compounds have influence on the efficiency of the extraction method used, regardless of the method applied (Azmir et al., 2013). Table 1 provides an overview of the extraction techniques and parameters used by the researchers who analyzed *Sambucus* fruit.

Table 1. Extraction parameters for *Sambucus* fruit

Extraction method	Solvent	Temperature	Extraction time (in total)	Reference	Applied in the paper
Maceration	Water 70% Ethanol 70% Acetone	Room temperature	5 days (in total)	Duymus et al., 2014; Vlachojannis et al., 2009	Duymus et al., 2014
Solid/liquid extraction	Acidified methanol (0.3% HCl, v/v)	Not mentioned	Until extraction solvent becomes colorless	Anton et al., 2013.	Anton et al., 2013
Pressured liquid extraction	Ethanol-water (80:20 v/v)	20 °C 100 °C	10 minutes	ASE 200 accelerated solvent extractor operator's manual, Document No. 031149, 1995.	Dawidowicz et al., 2006
Ultrasonic extraction	Acidified methanol (0.1% v/v formic acid)	Not mentioned	30 minutes	Rodriguez-Saona & Wrolstad, 2005.	Lee & Finn, 2007
Accelerated solvent extraction	1st hexane: dichloromethane (1:1, Hex, Dc) 2nd acetone:water: acetic acid (70:29.5:0.5, AWA)	70 °C 80 °C	20 minutes	Wu et al., 2004.	Wu et al., 2004
Ultrasonic extraction	MeOH acidified with 3% (v/v) formic acid, containing 1% 2,6-di-*tert*-butyl-4-methylphenol BHT	Cooled water bath	120 minutes	Escarpa & Gonzalez, 2000.	Petkovsek et al., 2007

4. Chemical analyses of berry extracts

Presence of the berry fruits in the human diet as a food and dietary supplement (Haminiuk et al., 2012; Seeram, 2006), also creates a need for the chemical analyses of berry extracts.

4.1. Spectrophotometric analysis

Spectrophotometric analysis is very important as a preliminary step for profiling and quantification of chemical compounds (Escarpa and Gonzalez, 2001). An important analytical disadvantage of direct spectrophotometric measurements could be attributed to the lack of selectivity, mainly because they overestimate the phenolic content (Robards and Antolovich, 1997); nonetheless, the spectrophotometric methods are still widely used in the analytical chemistry labs (Kanner et al., 1994; Sato et al., 1996).

4.1.1. Determination of total phenolic content (TPC)

The polyphenols are chemical compounds which possess the majority of the health benefits of berry fruits (Sidor and Gramza-Michalowska, 2014). They are target of many developed analytical procedures for studying the polyphenolic compounds. The methods used for total polyphenols determination are generally based on Folin-Ciocalteau's phenol reagent and spectrophotometric determination (Milivojevic et al., 2011; Rutz et al., 2012). One of the most cited papers for the determination of total phenolic content is published by Singleton et al. (1999). However, the most successful approaches for phenolic composition have been based on both, spectrophotometric and chromatographic (section 4.2.2.) methods.

Tables 2a and 2b provide an overview of the results for the TPC in *S. nigra* fruit. Results for different cultivars of *S. nigra* fruit are also available. There is no published result for TPC of *S. racemosa* fruit. Results in Table 2a and 2b are expressed as gallic acid equivalent – milligrams of gallic acid (g. a.) per 100 grams of berries.

Table 2a. Total phenolic content data for *S. nigra* fruit spectrophotometrically determined

Plant material	Extraction solvent	TPC in mg g. a./100 g	Reference
S. nigra fruit	Water	8974	Duymus et al., 2014
	70% ethanol	7594	
	Methanol	4917	
	70% acetone	8206	
	Acidified methanol	6399	
	Infusion	6715	

Table 2b. Total phenolic content data spectrophotometrically determined for different cultivars of *S. nigra* fruit in two growing seasons

Plant material	Extraction solvent	TPC in mg g.a./100 g	Reference
S. nigra fruit (cultivar Korsor 2004)	Acidified methanol	387	Lee and Finn, 2007
S. *nigra* fruit (cultivar	Acidified methanol	582	

Korsor 2005)			
S. nigra fruit (cultivar Haschberg 2004)	Acidified methanol	364	
S. nigra fruit (cultivar Haschberg 2005)	Acidified methanol	510	
S. nigra fruit	Acidified methanol	683.1	Mikulic-Petkovsek et al., 2016
S. nigra fruit (cultivar viridis)	Acidified methanol	268.8	

From the results in Table 2a, it is obvious that the extraction solvent has a key impact on the total phenolic content. To achieve the highest total phenolic content in the extract, maceration with water as an extraction solvent is recommended. Results in Table 2b show that the phenolic content in berries is affected by genetic differences (Zadernowski et al., 2005). Environmental conditions, soil composition, pollutions, light, temperature, stress conditions of plant during cultivation, also have an impact on the chemical composition (Tomas-Barberan and Espin, 2001). Identification and quantification of individual phenolic compounds is a topic in the section 4.2.2.

4.1.2. Determination of total monomeric anthocyanin content

Measurements of anthocyanin content, which contributes as the major colorant in berries and berry products, is an indicator of the quality of fresh and processed berry products (Wrolstad et al., 2005). Lee et al. (2005) validated and demonstrated the pH differential method as a simple, quick and accurate for measuring the total monomeric anthocyanin content of a sample. For the total anthocyanin determination there are simple spectrophotometric methods in use. One of the most cited papers on the determination of total monomeric anthocyanins spectrophotometrically is published by Giusti and Wrolstad (2000).

Table 3a shows results for total anthocyanins published by Duymus et al. (2014). Results in Table 3a are expressed as cyanidin-3-glucoside equivalent milligrams of cyanidin-3-glucoside per 100 grams (dry weight) of the extract. Table 3b shows results for total anthocyanins in two different cultivars of *S. nigra* expressed as mg cyanidin-3-glucoside (c-3-g) equivalent per 100 g of berries.

Table 3a. Total monomeric anthocyanins content data for *S. nigra* fruit spectrophotometrically determined

Plant material	Extraction Solvent	TPC in mg c-3-g/100 g	Reference
S. nigra fruit	Water	878.5	Duymus et al., 2014
	70% ethanol	1066.6	
	Methanol	408.6	
	70% acetone	651.1	
	Acidified methanol	600	
	Infusion	734.2	

Table 3b. Total monomeric anthocyanins content data spectrophotometrically determined for different cultivars of *S. nigra* fruit

Plant material	Extraction solvent	TPC in mg c-3-g/100 g	Reference
S. nigra fruit (cultivar Korsor 2004)	Acidified methanol	176	Lee and Finn, 2007
S. *nigra* fruit (cultivar Korsor 2005)	Acidified methanol	343	
S. nigra fruit (cultivar Haschberg 2004)	Acidified methanol	170	
S. nigra fruit (cultivar Haschberg 2005)	Acidified methanol	268	

The highest total monomeric anthocyanins content in extract is obtained when 70% ethanol is used as the extraction solvent for maceration of berries. As well as for the TPC, total monomeric anthocyanins content in extracts is affected by genetic differences (Zadernowski et al., 2005). Black elderberry fruit is rich in biologically active components, primarily polyphenols where anthocyanins belong (Sidor and Gramza-Michalowska, 2014). Cultivar Korsor of *S. nigra* is the richest in total monomeric anthocyanins content in fruit. The identification and quantification of them is of high importance. For that purpose, HPLC analyses are described in section 4.2.3.

4.1.3. Determination of antioxidant activity

Phytochemicals such as phenolic acids, flavonols and anthocyanins are responsible for the antioxidant activity of the fruits (Anton et al., 2013). The antioxidant characteristics of *S. nigra* fruit extracts are in correlation with phytochemicals (Pietta et al., 1992; Rice-Evans et al., 1996).

a) Determination of $ABTS^{\cdot+}$ radical cation scavenging activity

This assay determines the capacity of elderberry extracts to scavenge the $ABTS^{\cdot+}$. Relatively stable blue/green $ABTS^{\cdot+}$ is converting into a colorless product. Discoloration directly reflects the amount of $ABTS^{\cdot+}$ that has been scavenged, and can be measured spectrophotometrically. Trolox equivalent antioxidant capacity (TEAC) is calculated by comparing the scavenging capacity of the tested antioxidant to that of Trolox (Badarinath et al., 2010; Duymus et al., 2014) (Table 4a).

Table 4a. Antioxidant activity of *S. nigra* fruit extracts determined using $ABTS^{\cdot+}$ assay

Plant material	Extraction solvent	mM TroloxL^{-1}	Reference
S. nigra fruit	Water	1.85	Duymus et al., 2014
	70% ethanol	1.52	

Methanol	1.0
70% acetone	1.96
Acidified methanol	0.89
Infusion	1.23

Determination of $ABTS^{\cdot+}$ radical cation scavenging activity was also done (Mikulic-Petkovsek et al., 2016). The results are provided according to the published method of (Re et al., 1999). Results for antioxidant activity of two different black elderberry fruits are shown in Table 4b and expressed as mM Trolox equivalents per kilogram of berries (Mikulic-Petkovsek et al., 2016).

Table 4b. Antioxidant activity of *S. nigra* fruit extracts determined using $ABTS^{\cdot+}$ assay (mM Trolox/kg)

Plant material	**$ABTS^{\cdot+}$ assay**
S. nigra fruit	36.5
S. nigra fruit (cultivar viridis)	3.2

b) DPPH˙ radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH˙) radical is a stable radical with absorption maximum at 517 nm. Method using DPPH˙ radical for determining of scavenging activity is well known (Brand-Williams et al., 1995). The ability of polyphenolic compounds to act as free radical scavengers against DPPH˙ radical can be expressed as IC_{50} value or % of the residual DPPH˙. IC_{50} values are defined as the concentration required to scavenge 50% of the available free radicals; lower values are the indicators of higher radical scavenging activity.

Duymus et al. expressed their results for DPPH˙ radical scavenging activity as IC_{50} values (µg mL^{-1}). They found that the 70% acetone extract of *S. nigra* fruit was the most active with IC_{50}=117 µgmL^{-1}. The second most active extract was the water extract with IC_{50}=123 µgmL^{-1}. They investigated only these two extracts of *S. nigra* fruit with full details, and the others put results in the following order: 70% acetone > water > 70% ethanol > infusion > acidified methanol > methanol (Duymus et al., 2014). Positive control was ascorbic acid with IC_{50}=8 µg mL^{-1} (94% inhibition of DPPH˙).

The results in Table 2a may be related to the results for antioxidant activity determined by DPPH˙ method. Namely, the antioxidant activity of phenolic compounds shows the neutralization of free radicals initiating oxidation process or the termination of radical chain reaction. It can be concluded that higher total phenol content of extracts contributes to its higher antioxidant activity. However, these two properties are not directly correlated (Dawidowicz et al., 2006). This statement can be confirmed for the pure compounds, but not for plant extracts (Moure et al., 2001).

Table 5 shows results published by Anton et al. (2013) and Dawidowicz et al. (2006) which are arranged to show percentage of inhibition of DPPH˙. Also, Dawidowicz et al. (2006) consider the influence of the temperature at which the extraction of *S. nigra* fruit was carried out.

Table 5. Percentage of inhibition estimated by means of DPPH˙ method

Plant material	Extraction Temperature, °C	IC_{50}, μgml^{-1}	Reference
S. nigra fruit	Not mentioned	63.26	Anton et al., 2013
S. nigra fruit	20 °C	50.25	Dawidowicz et al., 2006
S. nigra fruit	100 °C	67.69	

The difference in the antioxidant activity of the same extract as a function of temperature is due to the different content of phytochemicals in it. Precisely, concentration of rutin, isoquercitrin and astragalin in the extracts increases with the increase of the extraction temperature (Dawidowicz et al., 2006).

c) Inhibition of β-carotene/linoleic acid co-oxidation

This spectrophotometric method for the determination of antioxidant activity is based on the ability of extracts to decrease oxidative losses of β-carotene in a β-carotene/linoleic acid emulsion (Tag et al., 1984; Velioglu et al., 1998). Results for this assay obtained by Dawidowicz et al. (2006) are presented in Table 6.

Table 6. Percentage of inhibition estimated by β-carotene/linoleic acid method

Plant material	Extraction temperature °C	Percentage of inhibition	Reference
S. nigra fruit	20 °C	3.87	Dawidowicz et al., 2006
	100 °C	6.63	

Similar to DPPH˙ assay results, results for inhibition of β-carotene/linoleic acid co-oxidation assay showed dependence on temperature. The explanation is the same - higher concentrations of different phytochemicals in the extracts at higher extraction temperature (Dawidowicz et al., 2006).

4.2. HPLC analyses

Identification and quantification of individual chemical components in *Sambucus* fruit is of great importance. There are many analytical procedures with this topic described in the literature. Almost all of them are based on high performance liquid chromatography (HPLC). High performance liquid chromatography (HPLC) is a versatile, robust, and widely used technique for the isolation of natural products (Cannell, 1998). This technique gained popularity as the main choice for identification and quantification of chemical components from plant material, among other analytical techniques (Fan et al., 2006). Usually, a reversed-phase C_{18} column, an UV-VIS detector and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B), are the chromatographic conditions for the HPLC methods (Gonzalez-Molina et al., 2012; Jakobek and Seruga, 2012; Sellappan et al., 2002;

Veberic et al., 2009). For accurate peak identification, procedures based on LC-MS, HPLC-PDA and HPLC-ESI-MS are developed (Osorio et al., 2012; Tamer 2012; Veberic et al., 2009).

4.2.1. Individual carbohydrates and organic acids – HPLC-PDA

Sugars and organic acids are primary metabolites of *S. nigra* fruit. Their concentrations are important in processing because sugars can be added into final product and organic acids cannot be added afterwards (Veberic et al., 2009).

Sample preparation and detailed description of method is published by Sturm et al. (2003). Veberic et al. (2009) used this method for the analysis of individual carbohydrates and organic acids in *S. nigra* fruit. The column used was Gemini C_{18} (150 × 4.6 mm, 3µm; Phenomenex) operated at 25 °C. As elution solvents 1% formic acid in twice distilled water (A) and 100% acetonitrile (B) were used. The concentrations of individual sugars and organic acids for two cultivars and three selections of *S. nigra* fruit are shown in Table 7 (Veberic et al., 2009).

Table 7. Concentrations of individual sugars and organic acids in *S. nigra* fruit of two cultivars and three selections (g kg^{-1} of fruit weight)

***S. nigra* cultivar/selection**	**Sucrose**	**Fructose**	**Glucose**	**Citric acid**	**Malic acid**	**Shikimic acid**	**Fumaric acid**
Haschberg	1.21	33.99	33.33	4.81	1.10	0.18	0.29
Selection 13	0.47	44.14	42.42	3.11	1.10	0.16	0.14
Selection 14	0.48	45.30	45.17	3.39	1.31	0.14	0.10
Selection 25	1.68	52.25	50.23	3.09	0.97	0.93	0.18
Rubini	1.38	44.12	41.93	3.08	1.02	0.24	0.13

These results were compared with those of other plant species. Black elderberry fruit contains moderate amounts of sugars compared to apple (115-183 g kg^{-1} fruit weight (FW)), and significantly lower amounts of total sugars than sweet cherry (150-230 g kg^{-1} FW). The content level of sugars in sour cherry (90 g kg^{-1} FW) is similar to *S. nigra* fruit (Veberic et al., 2009).

When it comes to organic acids, the total organic acids concentration of all cultivars/selections of *S. nigra* fruit is lower than in apple (6-14 g kg^{-1} FW) and sweet cherry (3.5-8.2 g kg^{-1} FW) (Veberic et al., 2009).

4.2.2. Individual phenolic compounds

Profiling the phenolic content is necessary to examine process-related variability of phenolic composition. The reason why HPLC has been the method of choice is because of its versatility, precision, and relatively low cost. Most frequently, the method is used on the reversed-phase C_{18} or C_8 columns in

conjunction with aqueous mobile phases and methanol, acetonitrile buffers as modifiers (Escarpa and Gonzalez, 2001).

a) HPLC-DAD-MS

Samples were prepared according to the protocol described by Mikulic-Petkovsek et al. (2013) with some modifications. Mikulic-Petkovsek et al. (2015) used this method for the determination of *Sambucus* fruit phenolic composition. The column was a Gemini C_{18} (150 × 4.6 mm, 3 μm; Phenomenex) set on 25 °C. The mobile phase A was aqueous 0.1% formic acid and B 0.1% formic acid in acetonitrile. Samples were eluted according to a gradient described by Wang et al. (2002). The results for qualitative and quantitative analysis of phenolic composition from *S. nigra* (2 different cultivars) and *S. racemosa* fruit are presented in Table 8 (Mikulic-Petkovsek et al., 2015).

Table 8. Phenolic compounds of two different varieties of *S. nigra* and *S. racemosa* fruit. The quantified compounds are expressed as mg kg^{-1} FW

Chemical compound	Fruit of *Sambucus* species			Reference
Cinnamic acid derivatives	*S. nigra* (nigra)	*S. nigra* (viridis)	*S. racemosa* (miquelli)	
3-*O*-Caffeoylquinic acid	88.41	38.85	-*	Mikulic-Petkovsek et al., 2015.
4-*O*-Caffeoylquinic acid 1	28.68	-	-	
4-*O*-Caffeoylquinic acid 2	-	-	4.71	
5-*O*-Caffeoylquinic acid 1	153.80	85.25	150.99	
5-*O*-Caffeoylquinic acid 2	40.13	-	-	
3-Feruloylquinic acid	18.78	16.38	-	
p-coumaric acid hexoside	32.22	9.35	7.56	
Caffeic acid hexoside	-	23.78	5.22	
3-*p*-Coumaroylquinic acid	11.94	19.77	-	
4-*p*-Coumaroylquinic acid 1	10.20	5.78	-	
4-*p*-Coumaroylquinic acid 2	×*	×	-	
Dicaffeoylquinic acid 1	3.43	0.62	0.49	
Dicaffeoylquinic acid 2	2.29	1.94	-	
Flavanols				
Epicatechin	63.71	-	12.43	
Procyanidin dimer 1	-	-	19.87	
Procyanidin trimer 1	-	-	×	
Procyanidin tetramer 2	-	-	×	
Flavonols				
Quercetin 3-*O*-glucoside	43.52	48.18	8.62	
Quercetin 3-*O*-rutinoside	313.30	341.75	2.02	
Quercetin acetylhexoside 1	4.66	29.81	3.55	
Quercetin acetylhexoside 2	×	-	-	
Quercetin hexoside pentoside 1	3.36	-	-	
Quercetin hexoside pentoside 2	×	-	-	
Kaempferol 3-*O*-rutinoside	4.2	52.26	-	
Kaempferol 3-*O*-glucoside	-	×	×	
Kaempferol acetyl hexoside 1	-	×	×	
Isorhamnetin 3-rutinoside	2.03	4.47	-	
Isorhamnetin hexoside 1	-	-	13.23	
Isorhamnetin hexoside 2	-	-	×	
Isorhamnetin acetyl hexoside 1	-	-	×	

Isorhamnetin acetyl hexoside 2	-	-	×
Flavanone			
Naringenin hexoside 1	×	×	-
Naringenin hexoside 2	×	-	-

* "-" – not detected; "×" – present

Mikulic-Petkovsek et al. (2015) performed detailed analysis of *Sambucus* fruit. Quercetin-3-rutinoside and 5-caffeoylquinic acid were the major phenolic compounds in all elderberry species and hybrids which they analyzed. Such a diverse chemical composition of phenolic compounds classifies *S. nigra* fruit as a good source of bioactive and health-promoting food ingredients (Wang and Bohn, 2012).

b) HPLC-DAD-ESI-MS-MS

Firstly, polyphenols were isolated by solid-phase extraction using a C-18 Sep-Pak mini column (Kim and Lee, 2005). This method was used by Lee and Finn (2007) for the determination of individual polyphenolic compounds in *S. nigra* fruit from two cultivars (Korsor, Haschberg) in growing seasons 2004 and 2005. The column was Synergi Hydro-RP 80A˚ (150 × 2 mm, 4 μm) coupled with 4.0 × 3.0 mm guard column (Phenomenex), operated at 25 °C. The results for qualitative and quantitative analysis are presented in Table 9 (Lee and Finn, 2007).

Table 9. Polyphenolic compounds of *S. nigra* fruit from two cultivars (Korsor, Haschber) in two growing seasons.

Chemical compound	**Fruit of *S. nigra* cultivars**		**Reference**
Cinnamic acid derivatives	*S. nigra* (Korsor) 2004/2005	*S. nigra* (Haschberg) 2004/2005	
3-caffeoylquinic Acid	11/44	7/9	
5-caffeoylquinic Acid	264/359	281/347	
4-caffeoylquinic Acid	12/25	16/19	
Flavonols			Lee & Finn, 2007.
Quercetin 3-rutinoside	465/426	727/956	
Quercetin 3-glucoside	95/149	39/52	
Kaempferol 3-rutinoside	7/11	7/12	
Isorhamnetin 3-rutinoside	3/22	7/7	
Isorhamnetin	Traces/3	1/Traces	

Cinnamic acids expressed as chlorogenic acid mg/kg FW, and flavonol glycosides are expressed as rutin mg/kg FW

When results for total phenol contents obtained by spectrophotometric method are compared with those obtained by HPLC (which is taken as a reference method because it is free of interferences), it can be observed that spectrophotometric method overestimates the phenolic content (Escarpa and Gonzalez, 2001).

4.2.3. Individual anthocyanins

Elderberry fruit is mostly used as a fresh food in daily diet, or being processed into other food products and dietary supplements. As a part of the food we eat, it is useful to know anthocyanins composition and contents, which represents the dominant share in elderberry fruit.

Most widely used tool for the identification and quantification of anthocyanins is reversed-phase HPLC coupled with photodiode array detection. The difference in polarity of individual anthocyanins allows their separation. The anthocyanins can be quantitated with an external standard (cyanidin-3-glucoside or any purified anthocyanin standard) (Lee et al., 2008).

a) HPLC-DAD

For analysis of individual anthocyanins Veberic et al. (2009) prepared samples and performed HPLC analysis accordingly to the method previously published by Marks et al. (2007). The column used was Gemini C_{18} (150 × 4.6 mm, 3µm; Phenomenex) operated at 25 °C. As elution solvents 1% formic acid in twice distilled water (A) and 100% acetonitrile (B) were used. The results for anthocyanins of two cultivars and three selections of *S. nigra* fruit expressed as mg cyanidin glucoside equivalents (CGE) per 1 kg of fruit are presented in Table 10.

Table 10. Concentrations of anthocyanins of two cultivars and three selections of *S. nigra* fruit (mg CGE/kg FW)

Chemical compound	**Fruit of *S. nigra* cultivars and selections**					**Reference**
Cinnamic acid derivatives	Haschberg	Selection 13	Selection 14	Selection 25	Rubini	
Cyanidin 3-sambubioside-5-glucoside	332.9	195.2	219.1	534.9	256.3	Veberic et al., 2009
Cyanidin 3,5-diglucoside	94.7	74.1	113.5	232.9	201.8	
Cyanidin 3-sambubioside	3527	2708	3467	5928	6308	
Cyanidin 3-glucoside	3317	4562	2214	2851	5864	
Cyanidin 3-rutinoside	96.3	29.8	14.9	25.2	22.5	

By observing the results, it can be said that black elderberry fruits are rich in anthocyanin content with the domination of cyanidin 3-sambubioside and cyanidin 3-glucoside compared to other fruit varieties (Veberic et al., 2009).

b) HPLC-MS-MS

Sample preparation and HPLC analysis for the determination and quantification of anthocyanins in *S. nigra* fruit was performed as published by Wu et al. (2004). The column used for separation was Zorbax SB-C_{18} A 250 × 4.6 mm. Mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol) were used as mobile phases in elution process. Obtained results were expressed as anthocyanidin glucoside equivalents (AGE).

Table 11. Concentrations of anthocyanins in *S. nigra* fruit (mg AGE/kg FW)

Chemical compound	Concentration of anthocyanins mg kg^{-1} FW	Reference
Cyanidin 3-sambubioside-5-glucoside	826	Wu et al., 2004.
Cyanidin 3,5-diglucoside	Nit quantified	
Cyanidin 3-sambubioside	5459	
Cyanidin 3-glucoside	7398	
Cyanidin 3-rutinoside	44	
Pelargonidin 3-glucoside	18	
Pelargonidin 3-sambubioside	Trace	

Wu et al. (2004) identified 7 anthocyanins. It should be mentioned that cyanidin 3-rutinoside, pelargonidin 3-glucoside and pelargonidin 3-sambubioside are chemical compounds in black elderberry identified for the first time by Wu et al. (2004).

c) HPLC-UV-MS-MS

For the identification of anthocyanins, Duymus et al. (2014) used this equipment and extracts obtained with different solvents for the extraction of *S. nigra* fruit. The column was 250 × 4.6 mm, 5µm octadecyl silica gel analytical column (Supelco) operating at 40 °C. Solvent A was formic acid/water (8.5/91.5, v/v), and solvent B was tetrahydrofuran/formic acid/acetonitrile/methanol/water (5/8.5/22.5/22.5/41.5, v/v/v/v/v) (Duymus et al., 2014). Details about applied method are published by Bermudez-Soto and Thomas-Barberan (2004). Results for identified anthocyanins in extracts of *S. nigra*

fruit are presented in Table 12. However, Duymus et al. (2014) did not quantify the identified anthocyanins.

Table 12. Anthocyanin composition in different *S. nigra* fruit extracts

Anthocyanin	A	B	C	D	E	F	Reference
Cyanidin 3,5-diglucoside	+	+	+	+	+	+	Duymus et al., 2014
Cyanidin-3-sambubioside-5-glucoside	+	+	+	+	+	+	
Cyanidin 3-glucoside	+	+	+	+	+	+	
Cyanidin 3-sambubioside	+	+	+	+	-	+	
Quercetin-3-rutinoside Na^+ adduct	+	+	+	+	-	+	

A – water extract; B – 70% ethanol extract; C – methanol extract; D – 70% acetone extract; E – acidified methanol extract; F – infusion; "+" – present; "-" – not detected.

d) HPLC-DAD-ESI-MS

For the detailed anthocyanin profile of *S. nigra* and *S. racemosa* fruit analysis, Mikulic-Petkovsek et al. (2014) have used the HPLC-DAD-ESI-MS method. Sample preparation and detailed procedure for applied method are described (Mikulic-Petkovsek et al., 2014). The column was a Gemini C_{18} (150 × 4.6 mm, 3 μm; Phenomenex) operated at 25 °C. The elution solvents were aqueous 0.1% formic acid in double distilled water (A) and 0.1% formic acid in acetonitrile (B). They calculated concentrations of anthocyanins from peak areas of the sample and the corresponding standards and expressed in mg per 1 kg of fresh elderberry fruits.

Tabela 13. Anthocyanin composition of different *Sambucus* species. Some of the identified compounds are quantified (mg/kg FW)

Chemical compound	Fruit of *Sambucus* species			Reference
	***S. nigra* (nigra)**	***S. nigra* (viridis)**	***S. racemosa* (miquelli)**	
Cyanidin-3-*O*-sambubiosyl-5-*O*-glucoside	421.9	0.9	40.8	Mikulic-Petkovsek et al., 2014
Cyanidin-3,5-*O*-diglucoside	59.1	0.2	Not quantified	
Cyanidin-pentoside-hexoside 4	10.8	×	1.7	
Cyanidin-3-*O*-galactoside	3.2	×	×	

Cyanidin-3-*O*-sambubioside	3444.8	10.3	13.6
Cyanidin-3-*O*-glucoside	1906.4	5.5	1.3
Cyanidin-3-*O*-rutinoside	93.6	×	×
Cyanidin-sambubioside-malonylglucoside	×	×	2.3
Pelargonidin-3-*O*-glucoside	Not quantified	×	×
Pelargonidin-3-*O*-sambubioside	Not quantified	×	×
Cyanidin-3-(*Z*)-*p*-coumaroylsambubioside-5-glucoside	×	×	25.9
Cyanidin-3-(*E*)-*p*-coumaroylsambubioside-5-glucoside	×	×	113.1
Cyanidin-3-*p*-coumaroyl-sambubioside	×	×	6.1

" ×" – not detected.

Sambucus fruit from different species shows difference in chemical composition. The species have different anthocyanin and polyphenolic components. But, the cultivars within each species have similar anthocyanin and polyphenolic profiles (Lee and Finn, 2007).

e) HPLC-DAD-ESI-MS-MS

This method was used by Lee and Finn (2007) for the determination of individual anthocyanin compounds in *S. nigra* fruit from two cultivars (Korsor, Haschberg) in growing seasons 2004 and 2005. The column was Synergi Hydro-RP 80A˚ (150 × 2 mm, 4 µm) coupled with 4.0 × 3.0 mm guard column (Phenomenex), operated at 25 °C. The results for qualitative and quantitative analysis are presented in Table 14 (Lee and Finn, 2007).

Table 14. Anthocyanin composition of fruit of different *Sambucus nigra* cultivars. Some of the identified compounds are quantified (mg kg^{-1} FW)

Chemical compound	**Fruit of *S. nigra* cultivars**		**Reference**
Anthocyanin	*S. nigra* (Korsor) 2004/2005	*S. nigra* (Haschberg) 2004/2005	
Cyanidin 3-sambubioside-5-glucoside	160/373	322/592	Lee & Finn, 2007.
Cyanidin 3,5-diglucoside	82/183	112/195	
Cyanidin 3-Sambubioside	1222/2691	2537/2681	
Cyanidin 3-glucoside	2537/4814	2046/3097	
Cyanidin	Not detected	Trace	

3-rutinoside		
Pelargonidin 3-glucoside	Trace	Trace
Cyanidin based Anthocyanin	Not detected	Not detected
Delphinidin 3-rutinoside	Not detected	Trace
Cyanidin 3-(*Z*)-pcoumaroylsambubioside-5-glucoside	Not detected	Not detected
Cyanidin 3-*p*-coumaroyl glucoside	Not detected	Not detected
Petunidin 3-rutinoside	Not detected	Not detected
Cyanidin-3-(*E*)-pcoumaroylsambubioside-5-glucoside	Not detected	Not detected
Cyanidin 3-coumaroyl sambubioside	Not detected	Not detected

Overall, values from the HPLC are higher than anthocyanin content obtained by the pH differential method. This phenomenon is observed before. Measurements examined by HPLC were even 2 times higher than the values obtained by pH differential method (Lee and Finn, 2007). Despite that, results obtained for total anthocyanin content by using both methods are significantly correlated (Lee et al., 2008).

4.2.4. Proanthocyanidins – HPLC-ESI-MS-MS

Proanthocyanidins are defined as oligomeric and polymeric flavan-3-ols. Polymerization degree describes the size of proanthocyanidins molecules (Porter, 1994). Procyanidins or prodelphinidins are names for those proanthocyanidins which contains (epi)catechin or (epi)gallocatechin as subunits. Anthocyanins and proanthocyanidins are one of the dominant phytochemicals in berries (Gu et al., 2004), both of which have been shown to be effective antioxidants (Wang et al., 1997).

Identification and quantification of proanthocyanidins in *S. nigra* fruit was done by Wu et al. (2004) according to the methods described previously by Gu et al. (2004). The column used for separation was Zorbax SB-C_{18} A 250 × 4.6 mm. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol). Results of Wu et al. (2004) about quantities of proanthocyanidins are presented in Table 15 and expressed as mg kg^{-1} FW.

Table 15. Concentrations of proanthocyanidins in *S. nigra* fruit (mg kg^{-1} FW)

Proanthocyanidins	Concentration of proanthocyanins mg kg^{-1} FW	Reference
Monomers	14.4	
Dimers	106.2	
Trimers	56.3	
4-6-mers	108	Wu et al.,
7-10-mers	Not detected	2004
>10-mers	Not detected	
Total	233	
Type	Procyanidins, prodelphinidins	

Conclusion

Sambucus nigra and *Sambucus racemosa* are two elderberry species with several cultivars within. *S. nigra* was much more attractive for examination than *S. racemosa*. Scientists described black elderberry in more details because of its use as a processed food or dietary supplement. Although *S. nigra* fruit is much richer with phytochemicals within, *S. racemosa* fruit should be examined more. Extraction conditions, extraction solvent(s), make a significant contribution to the chemical composition of elderberry extracts. So far, all extractions that have been made have been done with conventional solvents. Deep eutectic solvents are is use for extraction of plant material, so, scientific community is waiting for its application for the extraction of *Sambucus* fruit. Spectrophotometric analytical methods provide valuable information for characterization of plant material. For determination of chemical composition of individual sugars, organic acids, phenol components, anthocyanins, proanthocyanidins, there are different methods available. Different HPLC apparatus in combination with appropriate detector provides detailed simultaneously multicomponent analysis. *Sambucus nigra* and *Sambucus racemosa* fruit should be commercialized more. The author's suggestion is that, basic on the chemical composition, *Sambucus* fruit extract is a source for an active component in cosmetic products.

Acknowledgment

Vojkan Miljković wants to thank The Serbian Ministry of Education, Science and Technological Development for financial support through the Grant No. TR 34012.

Conflict-of-Interest Statement

There are no conflicts of interest in this review article.

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