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STANOVENÍ OBSAHU KYANIDU V RŮZNÝCH ČÁSTECH ROSTLINY BEZU ČERNÉHO

DETERMINATION OF CYANIDE CONTENT IN DIFFERENT PARTS OF ELDERBERRY PLANT

DIPLOMOVÁ PRÁCE

MASTER'S THESIS

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Zadání diplomové práce

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1. Zpracování literární rešerše k tématu DP
2. Stanovení obsahu kyanidu v různých částech bezu (kořen, list, květ, plod) pomocí molekulové absorpční spekrometrie
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ABSTRACT

Elderberry is an abundant shrub or a small tree widely spread in the northern hemisphere. Its fruit and flowers have attracted increasing interest from consumers, pharmaceutical and food industry thanks to their high content of minerals and bioactive compounds. However, elderberry also contain potentially harmful cyanogenic glycosides which can release toxic hydrogen cyanide. The aim of this experiment was to analyse and compare total cyanide content in different plant parts of numerous interspecific hybrids, which were created at University of Maribor. Investigated plants comprised 4 subspecies of *Sambucus nigra* and 18 interspecific hybrids giving in total 46 elderberry genotypes. Cyanide content was determined by picrate paper method which is based on reaction of picric acid with hydrogen cyanide. The colour change of the picrate paper resulting from the reaction was further analysed using spectrophotometry. The highest cyanide levels were found in leaves reaching up to 1155.9 ± 44.8 CN^- mg/kg, followed by inflorescences with maximum of 53.9 ± 3.8 CN^- mg/kg, shoots and berries with highest levels of 20.3 ± 1.3 CN^- mg/kg and 6.4 ± 0.0 CN^- mg/kg, respectively.

ABSTRAKT

Bez je hojně rozšířený keř vyskytující se především na severní polokouli. Díky vysokému obsahu minerálů a bioaktivních látek v plodech a květenství přitahuje nejen zájem konzumentů, ale také farmaceutického a potravinářského průmyslu. Bez však obsahuje také potenciálně škodlivé kyanogenní glykosidy, které mohou uvolňovat toxický kyanovodík. Cílem této práce bylo stanovit a porovnat celkový obsah kyanidů v různých částech rostlin bezu. Analyzované rostliny byly vyšlechtěny na Univerzitě v Mariboru a zahrnovaly 4 poddruhy *Sambucus nigra* a dalších 18 mezidruhových hybridů. K analýze obsahu kyanidu byla použita pikrátová metoda s následným spektrofotometrickým stanovením. Nejvyšší množství kyanidu bylo nalezeno v listech a dosahovalo hodnot až 1155.9 ± 44.8 CN^- mg/kg, dále následovala květenství s maximem 53.9 ± 3.8 CN^- mg/kg a poté výhonky a bobule s nejvyššími koncentracemi 20.3 ± 1.3 CN^- mg/kg a 6.4 ± 0.0 CN^- mg/kg.

KEYWORDS

Sambucus, elderberry, total cyanide content, picrate paper method, interspecific hybrids

KLÍČOVÁ SLOVA

Sambucus, bez, obsah celkových kyanidů, pikrátová metoda, mezidruhové hybridy

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DECLARATION

I declare that the diploma/bachelor thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma/bachelor thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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Bc. Kristýna Růžičková

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CONTENT

1 INTRODUCTION

Sambucus is a genus from the Adoxaceae family which comprises up to thirty plant species and numerous cultivars and hybrids. More familiar name for this abundant shrub or small tree native to the northern hemisphere is elderberry. Black elderberry (*Sambucus nigra*) is the most common type of elderberry followed by dwarf elderberry, red elderberry and American elderberry [1]. These wild growing species have been diffused in more diverse habitat including subtropical regions of Asia, North Africa and North America by birds and other animals spreading their seeds. Elderberry is simple to cultivate, highly available and all plant parts, especially elderberry berries and flowers, are a source of dietary phytochemicals, such as carbohydrates, lipids, phenolic compounds, terpenoids and alkaloids. Thanks to their antioxidant, anti-carcinogenic, immune-stimulating, anti-allergic, anti-viral and anti-bacterial properties there has been interest in elderberry since ancient times which is nowadays increasing together with its commercial value. For many centuries, elderberry has been used in folk medicine in the treatment of many diseases and ailments and later it has been also intensively used in food industry to produce pies, jellies, jams, ice creams, yogurts and different alcoholic beverages [2].

Elderberry flowers and berries contain not only beneficial but also potentially toxic compounds called cyanogenic glycosides which occur in approximately 2500 other plant species. Besides elderberry, cyanogenic glycosides accumulate in high levels in cassava, bamboo shoots, almonds or sorghum. These amino acid-derived secondary metabolites are important components of plant defence against herbivores [3]. Cyanogenic glycosides are composed from two main parts, an aglycone and a sugar moiety. The aglycone part consists of a nitrile group linked to an aliphatic, cyclic, aromatic, or heterocyclic moiety and the sugar moiety usually consists of glucose or a substituted glucose moiety. When the plant tissue is damaged by herbivore, specific enzymes can hydrolyse the resulting structures and the aglycone can be further degraded releasing toxic hydrogen cyanide (HCN) [4]. This phenomenon is called cyanogenesis. Cyanide poisoning in human generally causes diarrhoea, vomiting and weakness and the lethal dose of HCN was established to be 0.5 to 3.5 mg/kg of body weight [5].

The aim of this study was to determine total cyanide content of different plant parts of elderberry. The cyanide levels were determined and compared in samples from 46 elderberry genotypes, which included 4 subspecies of *Sambucus nigra* and 18 interspecific hybrids created at University of Maribor. Method utilised for the analysis was simple picrate paper method followed by spectrophotometric measurement.

2 THEORETICAL PART

2.1 Elderberry

Elderberry trees (*Sambucus*) belong to the family *Adoxaceae* and comprise shrubs and small trees growing in Europe, Asia, the Americas, and Africa [6]. The generic name *Sambucus* allegedly refer to a musical instrument called sambuca that was made from *Sambucus* twig and was introduced from the Orient into the Middle East by the Fifth century [7]. The stems of *Sambucus* tree were also used as kindling giving the name "Elder" (elderberry) which comes from the Anglo-Saxon word "aeld" meaning "fire". Archaeological findings indicate that people consumed elderberry fruit already in the Stone Age. Later, naturalists, healers and philosophers of Ancient Greece and Rome mentioned elderberry in their writings; Hippocrates called the elderberry his "medicine chest" and other well-known healers, such as Theophrastus, Dioscorides and Galen, referred to elderberry as one of nature's greatest healing plants [8], [9]. Before the discovery of antibiotics, elderberry represented one of the major ingredients for herbalists and pharmacists and until nowadays it has been used in non-conventional medicine mainly in form of extracts to treat cold, influenza and Herpes virus infections. In addition, elderberry fruit is used in food industry to produce juices, soft and alcoholic beverages, marmalades, or colorants [10].

2.1.1 Occurrence, species, and appearance

The *Sambucus* genus comprise up to 30 species and number of cultivars and hybrids. These tree-like shrubs with odd-pinnate leaves and fragrant flowers occur in northern hemisphere and prefer light to medium and heavy soils in semi-shade woodlands [2]. The most well-known species are black elderberry (*Sambucus nigra*) followed by dwarf elderberry (*Sambucus ebulus*), red elderberry (*Sambucus racemosa*) and American elderberry (*Sambucus canadensis*) [10]. Besides mentioned species that all grow in temperate and subtropical areas, some can be also found in the tropics (*Sambucus javanica*) [11]. Elderberry shrubs grow natively in the wild, however there are also elderberry cultivars bearing more abundant crops of larger and heavier berries. The most popular cultivars are "Sampo", "Samyl", "Alleso", "Korsor", and "Haschberg" [10].

The most common elderberry, black elderberry, is a European species with an oceanic to sub oceanic, cool-temperate and west Mediterranean range. Black elderberry commonly grows in western and central Europe as well as in North Africa, Scandinavia, and Great Britain [12]. This deciduous shrub growing to a height of 4–6 m have small white hermaphrodite flowers that are arranged in large corymbs and last from spring until summer. Berries have dark violet-black colour and a diameter of 3 to 8 mm. They grow in clusters and are only edible when fully ripe in late summer. The flowers and berries of black elderberry are shown in Figure 1. Other parts of the plant, such as green stems and branches, are not edible and are not recommended for human consumption [13].

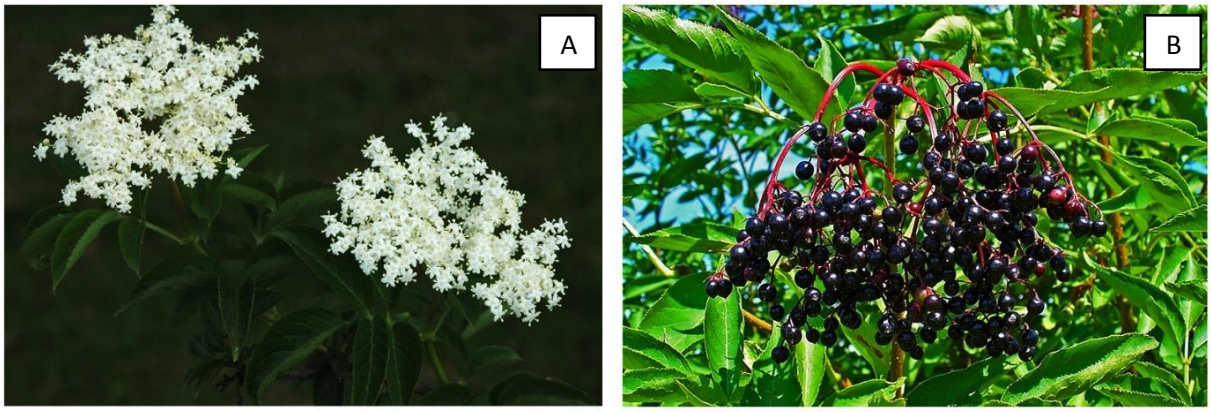


***Figure 1**: Black elderberry flowers (A) and fruit (B)* [14], [15].

2.1.2 Chemical composition of elderberry

Chemical composition of elderberry fruit and flowers consists of primary metabolites (carbohydrates, proteins, lipids), secondary metabolites (polyphenols) and other constituents (minerals, vitamins) [16], [17]. Occurrence of these compounds in elderberry is influenced by intrinsic factors (genetic factors, season of flowering and ripening, and the degree of fruit ripeness) since they affect the plant metabolism and thus the synthesis of secondary metabolites. Extrinsic factors such as exposure to different levels of radiation and wind, temperature, water availability, soil composition, and other agronomic factors also highly influence chemical composition of elderberry fruits and flowers [13].

2.1.2.1 *Carbohydrates*

Elderberry fruit contains approximately 18 % of carbohydrates of which 7 % is dietary fibre. Fibre fractions include pectin, pectic acid, protopectin and calcium pectinate [18]. Price of elderberry fruit on market is given by its total soluble solids which are related to levels of simple sugars present in the fruit. Different cultivars contain different amounts of sugars even in the same maturity stage, however in all cultivars the major sugars are glucose and fructose, followed by sucrose which is present in much lower amounts [13].

2.1.2.2 *Proteins*

The content of protein is 2.7–2.9 % in elderberry fruit, 2.4 % in flowers and 3.3 % in leaves. The protein of elderberry is a complete one. There are sixteen amino acids found in fruit and seven of them belong to the group of exogenous and relative exogenous amino acids. Nine amino acids are found in flowers and leaves [19].

2.1.2.3 *Lipids*

Lipids in elderberry fruit are found predominantly in the seeds, which contain high amounts of polyunsaturated fatty acids (~80 %) and low amounts of saturated fatty acids (<10 %). The n-6/n-3 ratio is of about 1, which agrees with recommended healthy fat/oil ratio [15]. The fatty acid profile of lipids is dominated namely by linolenic (40.7 g/100 g oil), linoleic (34.3 g/100 g oil) and oleic acid (13.8 g/100 g oil) [20].

2.1.2.4 *Organic acids*

Important component that gives elderberry fruit its pleasant acidic taste are organic acids. Elderberry fruit contain high levels of citric and malic acids and smaller amounts of tartaric, shikimic, and fumaric acids. On the other hand, elderberry flowers are rich in malic acid, following by citric, tartaric, shikimic, and fumaric acid [13].

2.1.2.5 *Antioxidant activity and polyphenols*

Antioxidants are molecules that slow down or inhibit the production of free radicals produced by oxidation processes. Free radicals are reactive and unstable molecules which can damage cells and cause various diseases [21]. Examples of free radicals are reactive oxygen species such as superoxide anions, hydroxyl radicals, alkoxyl radicals and peroxyradicals. Under physiological conditions, the human antioxidative defence system eliminates excess of these reactive species. However, the endogenous antioxidants are not complete without exogenous antioxidants such as vitamin C, vitamin E, carotenoids, and polyphenols. These molecules are important for various antioxidant processes and hence, there is constant need for exogenous antioxidants to prevent oxidative stress (a disequilibrium redox state in favour of oxidation) [22].

Polyphenols are secondary plant metabolites and the most important bioactive compound contributing to the antioxidant properties of elderberry. Polyphenols can be classified based on the number of phenol rings and their structural elements as seen in Figure 2. Flavonoids, which comprise anthocyanins and flavonols, followed by phenolic acids are the most abundant polyphenol groups in elderberry fruit responsible for its antioxidant properties [15].

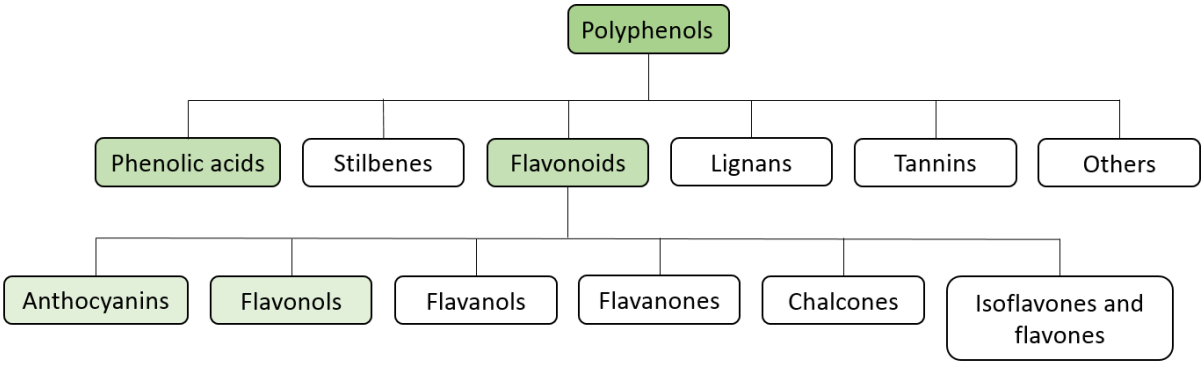


Figure 2: Classification of polyphenols based on their chemical structure. Adapted from [15].

2.1.2.6 *Flavonols*

Both elderberry fruit and flowers contain flavonols, namely quercetin-3-rutinoside (rutin), quercetin-3-glucoside, kaempferol-3-rutinoside, isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside and quercetin-3-6-acetylglycoside. The concentration of flavonols is high especially in elderberry flowers which contain up to 21.0 mg of rutin per g dry weight [15], [23].

2.1.2.7 *Anthocyanins*

Anthocyanins are a class of flavonoid compounds which are responsible for the attractive red and violet colours of many vegetables, fruits and flowers. The anthocyanins present in elderberry fruit are cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-sambubioside, cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-glucoside, and

pelargonidin-3-sambubioside [23]. The cultivars with highest content of anthocyanins are 'Mammut', 'Samdal', 'Sampo' and 'Samyl', while 'Finn Sam', 'Haschberg', 'Allesoe', but also wild elderberry have the lowest quantities. Total anthocyanin content varies almost threefold depending on the cultivar, for example 'Haschberg' was found to contain 664 mg of total anthocyanins/100 g, while 'Sampo' contained 1816 mg/100 g [24]. Opposed to elderberry fruit, which is especially rich in anthocyanins, elderberry flowers do not contain any [23]. Anthocyanins, as well as other flavonoids exhibit antioxidant, anticarcinogenic, immune-stimulating, antibacterial, antiallergic and antiviral properties and their consumption can prevent several degenerative diseases such as cardiovascular disease, cancer, inflammatory disease, and diabetes [25].

2.1.2.8 *Phenolic acids*

Eleven phenolic acids were found in elderberry flowers with the major phenolic acids 5-caffeoylquinnic acid and 1,5-di-caffeoylquinnic acid comprising over 70% of the total phenolic acid content [23].

2.1.2.9 *Cyanogenic glycosides*

Not all secondary metabolites in elderberry are beneficial. Various parts of elderberry tree especially leaves, seeds, bark and unripe fruit accumulate potentially toxic cyanogenic glycosides. Black elderberry contains substantial amounts of cyanogenic glycoside sambunigrin. Furthermore, elderberry contains lower amounts of its isomer prunasin and m-hydroxysubstituted glycosides, such as zierin and holocalin [24], [25]. These molecules are potentially toxic and life-threatening, because they can decompose into chemically reactive and toxic hydrogen cyanide. Excessive consumption of elderberry parts containing high level of sambunigrin may cause gastrointestinal disorders, including nausea, vomiting, weakness and dizziness [26].

2.1.2.10 *Vitamins and minerals*

Elderberry fruit contains B-group vitamins, A-group vitamins, tocopherols (vitamin E) and vitamin C. The content of vitamin C in fresh elderberry fruit is 6–35 mg/100 g [18]. Vitamins E and C are important antioxidants, and their content contributes to the antioxidant activity of elderberry fruit. Vitamin E transfers hydrogen atoms to radicals, delays hydroperoxides decomposition, scavenges singlet oxygen and metals complexes in the presence of ascorbate and therefore is considered as one of the most effective antioxidants. Vitamin C and E together produce a synergic antioxidant effect since vitamin C neutralizes tocopheroxy radicals and regenerates the α-tocopherol molecule (Figure 3). Additionally, vitamin C can scavenge oxygen and numerous free radicals. Pro-vitamin A (β-carotene) has both antioxidant and colorant properties [15].

Ash in elderberry fruit represents about 0.99 %, and consists of the following minerals: K, Ca, Fe, Mg, P, Na, Zn, Cu, Mn, Se, Cr, Ni, Cd [18]. The study on mineral content in various interspecific hybrids and different plant parts of elderberry showed that leaves contained the highest amounts of Ca, Mg, Mn, Zn, and Sr, while K and P are mainly found in fruit stalks. Fe and Al occurred in the highest amounts in roots and Cu in bark. The mineral content in elderberry fruit is comparable to other frequently consumed berries [27].

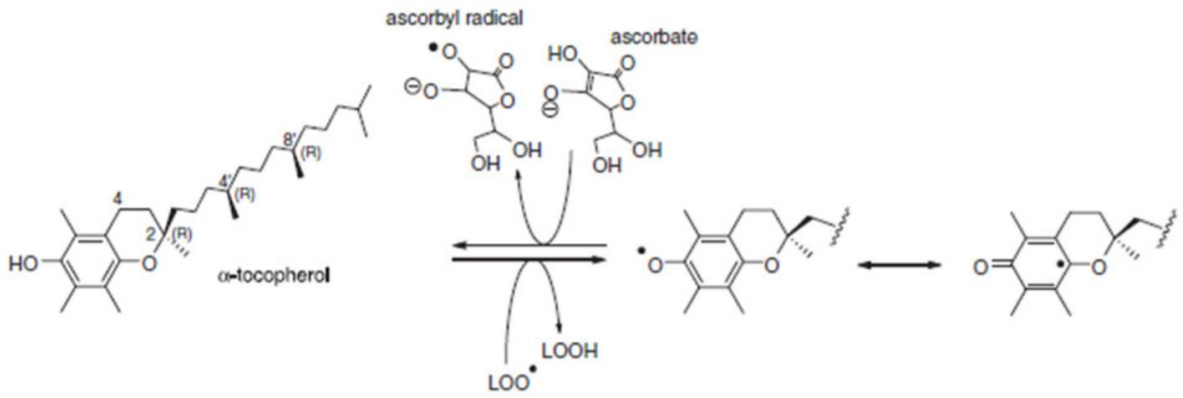


Figure 3: Antioxidant effect of vitamin E. α-Tocopherol reacts with a lipid hydroperoxyl (LOO•) radical. The resultant tocopheryl radical is resonance-stabilized and does not react with oxygen (unlike L• radicals) and it can be converted back to α-tocopherol by ascorbate [28].

2.1.3 Use of elderberry in food industry

In Europe, elderberry is cultivated for flowers and fruit production as well as for ornamental purposes. Both flowers and fruit are used in preparation of drinks such as wine, beer, or liquor. Elderberry flowers with their characteristic enjoyable aroma are often used as flavouring agents, in infusion, pastry products, non-alcoholic cordials and fermented beverages [29]. Elderberry fruit is also processed to concentrates, syrups, pies, ice creams, jellies, juices [23]. The waste by-product of juice production is elderberry pomace, which is used in the production of anthocyanin extracts and natural colorants. The production of elderberry fruit products deals with issues such as low stability of anthocyanins towards heat, oxygen, light, and increased pH values [15]. The storage conditions of elderberry fruit can affect its antioxidant and colorant properties. It was observed that the amount of total monomeric anthocyanins of elderberry juice concentrate stored at refrigerated temperatures was reduced by 14–22 %, while in the same concentrates stored at room temperature the losses reached 67–71 % [30]. In production of alcoholic beverages, the elderberry fruit undergoes alcoholic fermentation that causes changes in the content of phenolic compounds and anthocyanins, inducing colour changes. In study of Schmitzer et al., the concentration of polyphenols was higher in wine than in must. Due to the storage and aging of elderberry wine the total phenolic content fell by 21 % and total anthocyanins content lowered about 94 % inducing colour change [31]. Besides mentioned food products, elderberry fruit can be also utilized as animal feed and organic fertilizer [24].

2.1.4 Use of elderberry in medicine

In addition to food industry, flowers and fruits of black elderberry are also utilized for medicinal purposes [23]. These plant parts are often used in folk medicine to ameliorate symptoms of cold, fever, cough, nasal congestion, mucous discharge, influenza, and to strengthen the immune system. Elderberry flowers contain high levels of flavonoids and thus serve as diaphoretic, antipyretic and diuretic agents. Rutin, the main flavonoid in elderberry flowers, obturate the capillary walls, promote their flexibility and prevent infiltration of red blood cells and plasma outside the vessels. Elderberry flowers also possess anti-inflammatory and antibacterial properties and hence are used for gargling to treat sore throats or as compresses to

treat conjunctivitis. Elderberry flowers are dried and processed into infusions which can be applied internally or externally [24].

As well as flowers, elderberry fruit shows diaphoretic, antipyretic and diuretic properties. Furthermore, the fruit acts as a laxative and detoxifier and therefore is often utilised in herbal mixtures for slimming and due to its analgesic effect, it can be used also as an adjuvant painkiller against migraine, sciatica and neuralgic pains. Black elderberry fruit or flowers are nowadays used in developing of medicaments treating cold, flu and other infectious illnesses in the form of syrups, drops, tablets, capsules, lozenges, aerosols, emulsions or suspensions [24]. A very popular supplement sold as a prophylactic agent in many countries is the Sambucol preparation, patented in Israel by Dr. Madeleine Mumcuoglu [25]. Bark, root, stem and leaves have been used as medicine or food industry as well. Elderberry bark has been known for its diuretic and slimming properties and elderberry leaves can positively affect resistance to infectious diseases [24]. Elderberry leaves are applied to help against symptoms of rheumatism and to treat acne [32]. According to a 2010 report by the European Herb Growers Association, black elderberry (flowers and berries) was the most harvested medicinal plant exported for trade, tea and phytopharmaceutical production in Bulgaria and Romania and in 2011, black elderberry was also ranked as the 18th best-selling herbal dietary supplement on the medicine, food and mass market in the USA [25].

2.2 Cyanogenic glycosides

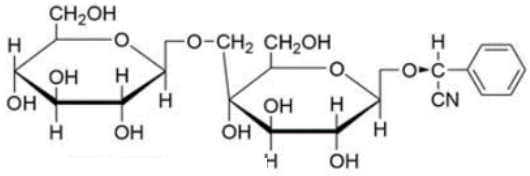
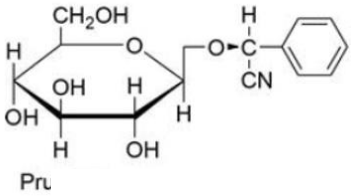
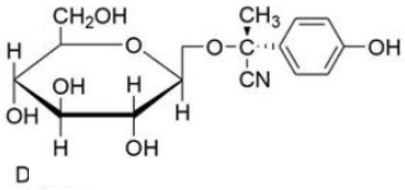
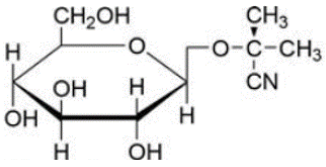
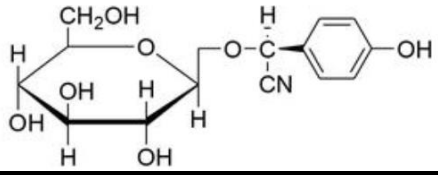
Elderberry contain natural plant toxins called cyanogens. When is cyanogenic plant material crushed during consumption or processing, cyanogens are hydrolysed to form toxic hydrogen cyanide (HCN). This process is known as cyanogenesis and it occurs in approximately 3000 species of plants representing more than 110 plant families. With their bitter taste, odour and toxicity cyanogens serve as defence agents against herbivores and pathogens. There are three groups of cyanogens: cyanogenic glycosides, pseudocyanogenic glycosides and cyanogenic lipids [33]. Cyanogenic glycosides are the most important and abundant cyanogens of many plants used either in human nutrition or as livestock feed. The majority of 75 known cyanogenic glycosides is found in dicetyledons belonging to families Fabaceae, Asteraceace, Euphorbiaceae, Passifloraceae, Poaceae and Araceae [34]. The main cyanogenic glycosides found in plants that are commonly used in human diet are amygdalin (almonds, stone fruit, pome fruit), dhurrin (sorghum), linamarin (cassava, lima beans, linseed, spinach), linustatin (cassava, linseed), lotaustralin (cassava, lima beans), prunasin (stone fruit, pome fruit), and taxiphyllin (bamboo shoots) [35].

2.2.1 Structure and biosynthesis

Cyanogenic glycosides are composed from two parts, aglycone and sugar moiety. Aglycone consists of a nitrile group linked to an aliphatic, cyclic, aromatic, or heterocyclic moiety and is biosynthesized from amino acids in three-stepped process. Firstly, amino acid precursor molecules are converted to aldoxime intermediates catalysed by an enzyme from cytochrome P450 family. Another enzyme of the same family then converts aldoxime molecules into cyanohydrins which are glycosylated in the last step by an UDP glucosyltransferase to form cyanogenic glycosides. Cyanogenic glycosides prunasin, sambunigrin, and amygdalin are derived from phenylalanine, while dhurrin and taxiphyllin are derived from tyrosine. Valine,

isoleucine, and leucine are precursors of linamarin, lotaustralin, and heterodendrin, respectively [36]. The sugar part usually consists of a monosaccharide β-D-glucose or a substituted glucose moiety. Besides glucose other sugars as allose, apiose, arabinose, rhamnose, and xylose occur [37]. Table 1 shows the structure details of the major cyanogenic glycosides found in human food. These compounds are highly structurally related. Dhurrin and taxiphyllin are stereoisomers (epimers), as are prunasin and sambunigrin. If a single sugar is removed, amygdalin converts to prunasin and linustatin to linamarin [35].

Table 1: Structure and occurrence of main cyanogenic glycosides found in food [34], [38].

Name	**Food**	**Structure**
Amygdalin	Stone fruit, pome fruit	
Prunasin	Ferns	
Dhurrin	Sorghum	
Linamarin	Cassava, lima beans, linseed	
Taxiphyllin	Bamboo shoots	

2.2.2 Cyanogenesis

Cyanogenic glycosides are stored in vacuoles, mostly in leaf tissues. Majority of plants containing cyanogenic glycosides produce also endogenous hydrolysing enzymes β-glycosidases and α-hydroxynitrilases [39]. β-glycosidases are stored in the apoplastic space attached to cell walls in the cytoplasm, vesicles, or chloroplast while hydroxynitrile enzymes are accumulated in cytoplasm. Once the plant tissue is disrupted by crushing, chewing, herbivory or disease, the enzymes and cyanogenic glycosides are brought together and cyanogenic glycosides are degraded into cyanohydrins, HCN, and ketones. Depending on the type of the cyanogenic glycoside, HCN is released in a two-step or three-step process which is illustrated in Figure 4. Linamarin (A) is cyanogenic monoglycoside which firstly forms unstable α-hydroxinitriles due to β-glucosidases activity (1) and then dissociate into HCN and carbonyl compound either spontaneously or with presence of α-hydroxynitrile lyases (2). Amygdalin (B) is cyanogenic diglycoside that firstly reacts with amygdalin hydrolase (1) and releases one of its D-glucose moiety. The resulted prunasin molecule reacts with prunasin hydrolase (2), releases the other molecule of D-glucose and produces mandelonitrile. Mandelonitrile is a benzaldehyde cyanohydrin that is degraded by mandelonitrile lyase (3) into benzaldehyde and HCN [36].

Generated HCN is toxic for plants themselves and therefore is detoxified in two-stepped process. Firstly, β-cyanoalanine synthase catalyses conversion of HCN and cysteine into β-cyanoalanine and H_2S. In the second step, NIT4 family nitrilase catalyses conversion of the toxic *β*-cyanoalanine into ammonia, aspartic acid and asparagine. All mentioned products can be further utilized in plant metabolism [40], [41]**.**

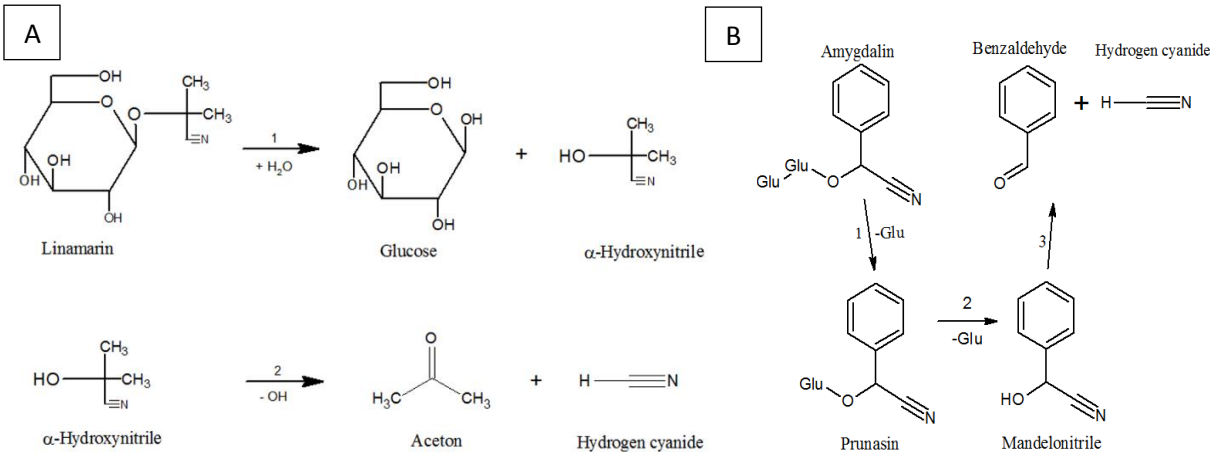


Figure 4: The process of HCN release from linamarin (A) and amygdalin (B) [40].

2.2.3 Function of cyanogenic glycosides in plants

Main function of cyanogenic glycosides in plants is defence against herbivore. The highest levels are found in reproductive organs (flower, fruit, and seed) and in young leaves, where they are most needed for plant protection [42]. Except chemical defence cyanogenic glycosides have additional physiological functions that contribute to better plant phenotypic plasticity during its development and under environmental stress. They might be involved in modulating oxidative stress by scavenging reactive oxygen species (e.g. H_2O_2 in chlorotic leaves), suggested by studies on *Olinia* [43]. Studies on *Hevea brasiliensis* (rubber tree) have shown

that cyanogenic diglucosides function as a transport and storage form of renewable nitrogen, carbon and glucose for both germination and latex production [44].

2.3 Cyanogenic plant foods

Concentrations of cyanogenic glycosides in plants vary depending on the cultivar, plant age, soil condition, fertilizer application, weather, and other factors. Cyanogenic content usually increases when growth is limited by environmental factors such as light, temperature, or drought. This finding has been explained in three ways: (a) cyanogenic glycosides are concentrated in a smaller amount of plant tissue, (b) due to delayed growth, the plants are in phenological manner younger, or (c) there is active upregulation at the transcriptional level which could be either an adaptation to protect the plant tissue from herbivores or a mechanism for moderating oxidative stress [45]. Content of cyanide in different plants and their parts used for consumption is shown in Table 2.

Table 2: Amounts of HCN in different plants given in mg.kg^{-1} of fresh weight [34].

Plant or plant part	HCN in mg.kg^{-1} of fresh weight	Plant or plant part	HCN in mg.kg^{-1} of fresh weight
Cassava		**Linseed**	200−380
Leaves	650−1040	**Stone fruit**	
Whole Tuber	550	Bitter Almonds	2800−3100
Tuber Bark	840−2450	Apricot Kernel	3200
Tuber Pulp	100−330	Cherry Kernel	3540
Bamboo		**Sorghum**	
Unripe Shoots	3000	Young Leaves	600
Tips of Unripe Shoots	8000	Germinating Plants	2400
Passion fruit		**Legumes**	
Unripe Fruit	700	Lima Bean	100−4000
Ripe Fruit	100	Green Bean	20

2.3.1 Cassava

Cassava (*Manihot esculenta*), also called manioc, mandioca, tapioca, or yuca, is a major staple root crop in many tropical and subtropical developing countries, especially in West Africa [46]. Portuguese explorers introduced cassava from South America to Africa during the sixteenth and seventeenth centuries [47], [38] and nowadays is cassava grown in more than 90 countries and ranked as the 6th most important source of energy in human diets on a worldwide basis. Cassava is a nutritionally strategic famine crop because its mature roots (Figure 5) can survive long periods without rain and still retain their nutritional value. Cassava roots are also a valuable source of calories, containing 80 % to 90 % carbohydrate on a dry weight basis of which 80 % is starch. Cassava leaves are a good source of protein, they are rich in minerals such as iron, zinc, manganese, magnesium, and calcium; vitamins B1, B2, and C and carotenoids [46].

Cassava contains potentially toxic levels of cyanogenic glucosides in all its tissues except seeds, made up of linamarin (>90% total cyanogen) and lotaustralin (<10% total cyanogen). Leaves contain the highest amounts while roots contain approximately 20 times less depending on the variety. Sweet cultivars of cassava usually contain only 15−50 mg/kg total cyanogen of fresh weight. These varieties with low cyanogen content can be processed only by peeling and cooking, whereas bitter varieties of cassava require more extensive processing [47], [48]. The root can be boiled and eaten as whole root, or it can be processed into flour, tapioca, and other types of foods. Cassava leaves are used for soup or sauce, cassava roots and peels are also consumed by animals [38].

2.3.2 Bamboo shoot

Bamboo shoot (Figure 5) is an edible plant part with one of the highest contents of HCN exceeding that of apricot, bitter almonds, and cassava. They are used in various Asian dishes and sold in markets in sliced forms, fresh, fermented, and canned version. Young bamboo shoots are the most favourite since they have high fibre content, they are rich in minerals, have suitable amount of glucose and low levels of fat. Except cyanogenic glycosides, there are other secondary metabolites in bamboo which can be used as precursors in pharmaceutical industry [49]. Bamboo shoots can contain from 1000 mg HCN/kg up to 8000 mg HCN/kg of fresh weight, but the content significantly decrease with harvesting and processing. The cyanogenic glycoside present in bamboo shoot is taxiphyllin, which quickly decomposes when exposed to boiling water [47].

2.3.3 Sorghum

Sorghum (*Sorghum bicolor L.*) is the most produced cereal in the world after wheat, rice, maize, and barley and presents an important food crop mainly in Asia and Africa. Sorghum grains (Figure 5) are an attractive source of nutrients and bioactive compounds for the human diet. They are composed from starch, which is digested at slower pace than of other cereals. Sorghum grains contain low digestibility proteins, unsaturated lipids, several minerals (phosphorus, potassium, and zinc), B-complex vitamins (thiamine, riboflavin, and pyridoxine) and fat-soluble vitamins (D, E, and K). Furthermore, red, brown, and black coloured varieties, have a high content of phenolic compounds, which are beneficial to human health. However, in Western countries such as the United States, Australia, and Brazil, sorghum is developed and cultivated primarily for animal feeding [50].

Cyanogenic glucoside present in sorghum is dhurrin. The levels of dhurrin are the highest in young plants and decrease as the plant matures. In three-day old seedlings, dhurrin concentration can be as high as 6%, with high concentration of dhurrin localized in the growing shoot tips where it may constitute up to 30% of the total shoot dry weight [48].

Figure 5:Cassava tuber roots (A), bamboo shoots (B) and sorghum seeds (C) [51], [52], [53].

2.3.4 Fruits and seeds

Majority of fruits and fruit kernels contain cyanogenic glycoside amygdalin. Levels of amygdalin can differ even in the same species due to fruit varieties, cultivation practices, environmental conditions and moisture content. Two important sources of amygdalin are apples and apricots. Apples are rich sources of vitamins and other nutrients however apple seeds contain high levels of amygdalin, ranging from 1000 to 4000 mg/kg [43]. Apricots (*Prunus armeniaca* L.) is a nutritious fruit cultivated in the Middle Asia, Africa, America, and Europe [43]. Apricot is known as the 'golden fruit' due to their nutritional value and medicinal properties. The fruit is rich in bioactive phytochemicals such as carotenoids, flavonoids, phenolics, and antioxidants and is considered as a functional food [53]. Apricots can be consumed raw or dried, however their kernels (Figure 6) are mostly processed before consumption. There are two varieties of apricot kernels: bitter and sweet. Bitter apricot kernels contain high levels of amygdalin compared to sweet apricot kernels with low level of cyanogens that are safe for human consumption. The concentration of HCN in apricot kernels varies widely (49–4000 mg/kg), depending on whether skin on or off varieties is surveyed [43].

2.3.5 Nuts and seeds

Almond (*Prunus amygdalus*) is an important nut growing worldwide in hot-arid Mediterranean climate regions. Sweet almond seeds (Figure 6) are rich source of monosaturated fatty acids, riboflavin, magnesium, manganese, and vitamin E. They are used by patients suffering from diabetes mellitus, because they contain low levels of carbohydrates and almond flour is consumed by people with wheat allergies and celiac disease, since it is gluten-free [41].

The predominant cyanogenic glycosides in almonds are amygdalin, prunasin and linamarin. Sweet almond seeds contain much lower amounts than the seeds of bitter almonds which contain up to 5% of amygdalin (approximately 1 mg HCN per seed). Lethal dose for children is 10−15 of bitter almond seeds and 50−60 of bitter almond seeds for adults [41].

Figure 6: Apricot and apricot kernels (A), almond and almond seeds (B) [55], [56], [57], [58].

2.4 Legislation

An extensive study on the identification and characterization of the risks and health hazards of cyanide was conducted by The European Food Safety Authority (EFSA). It was reported that the lethal dose is 0.5 to 3.5 mg/kg of body weight. In Europe, the presence of cyanide in food, beverages, and additives is regulated by various standards. A maximum level of HCN of 50 mg/kg has been set up in nougat, marzipan and their substitutes or similar products, 5 mg/kg in canned pitted fruits and 35 mg/kg and a maximum HCN content of 7 g/hL at 100% volume alcohol in fruit marc brandy and stone fruit brandy. Cyanogenic glycosides are mentioned in the legislation of several countries including the United Kingdom, Germany, Australia, and New Zealand [5].

2.5 Toxicity of HCN

Low doses of HCN can be efficiently metabolized in mammals. Enzyme rhodanese located in mitochondria mediates conversion of HCN into thiocyanate which is excreted in the urine. Required sulphur donors for this reaction are usually provided from the dietary sulphur amino acids cysteine and methionine [41]. However, consumption of food products with high amounts of cyanogenic glycoside can lead to various health complications such as acute intoxication, chronic toxicity, neurological disorders, growth retardation, and goitre [43].

2.5.1 Acute toxicity

Oxidative phosphorylation is a process in which electron carriers transfer electrons from NADH or $FADH_2$ to O_2 leading to production of essential cellular energy sources in the form of adenosine triphosphate (ATP) [59]. Cytochrome *c* oxidase is the terminal enzyme of the mitochondrial electron transport chain which is essential for reduction of oxygen to water in the fourth complex of oxidative phosphorylation. HCN competitively binds to ferric iron (Fe^{3+}) in the haem moiety of the oxidised form of cytochrome *c* oxidase and inhibits the enzyme [60], [61]. This leads to lower utilization of oxygen and increase in anaerobic metabolism, resulting in excess of lactic acid and metabolic acidosis, and consequently in cell death through energy deprivation [59]. The heart and brain are especially sensitive to HCN acute toxicity since they need constant supply of oxygen and ATP generated from aerobic metabolism. Except cytochrome *c* oxidase HCN inhibits about 40 other enzymes, including several other important metalloenzymes, such as alkaline phosphatase, carbonic anhydrase, catalase, peroxidase, ascorbic acid oxidase, xanthine oxidase and succinic dehydrogenase [60].

The minimal lethal dose of HCN in humans is usually cited as 0.5 mg/kg body weight. With high oral doses symptoms can appear in several minutes after intake. Symptoms of acute toxicity include nausea, vomiting, giddiness, headache, heart palpitations, hyperpnoea, then dyspnoea, unconsciousness, and uncontrollable muscle contractions, followed by death [59]. Acute cyanide poisoning is treated in hospital with specific antidotes, that needs be given as soon as possible after exposure [62]. There are several antidotes such as The Cyanide Antidote Kit (CAK), 4-dimethylaminophenol (4-DMAP), dicobalt edetate (Kelocyanor), and hydroxocobalamin (Cyanokit), differing in mechanisms of action and diverse toxicological, clinical, and risk–benefit profiles [63].

2.5.2 Konzo

Konzo is defined by the World Health Organization (WHO) as a distinct upper motor neuron disease characterized by the abrupt onset of a symmetric, nonprogressive, but permanent paraparesis (partial paralysis of both legs that could be seen in Figure 7) [64]. The disease is associated with prolonged high dietary intake of cyanogens from insufficiently processed roots of bitter cassava in combination with a protein-deficient diet low in sulphur amino acids such as cysteine and methionine. The outbreaks of Konzo have occurred in Cameroon, Mozambique, Tanzania, the Central African Republic, and the Democratic Republic of Congo mostly affecting children and women of childbearing age. The first cases of Konzo were described in 1939 and 6,788 cases of Konzo were reported up to 2009. However, outbreaks in past decades rarely received attention from the media and local health authorities. The number of people affected by Konzo is underestimated, since they live in the poorest parts of remote rural areas

of Africa. Unofficial reports suggest that there have been hundreds of thousands of cases with the majority happening in the Democratic Republic of Congo [65].

Figure 7: Two subjects affected by Konzo disease in Democratic Republic of Congo [66].

2.5.3 Other diseases

Tropical ataxic neuropathy (TAN) is neurological syndrome affecting the mouth, eyesight, hearing or gait of older people. TAN, as well as Konzo is attributed to chronic cyanide exposure from consumption of cassava derived foods. Consumption of insufficiently processed cassava also leads to worsen goitre and cretinism due to iodine deficiency [35].

2.6 Food processing technologies reducing cyanogenic glycosides

Cyanogenic glycosides in food can be reduced by several processing technologies such as peeling, drying, grinding, boiling or cooking, soaking and fermentation. These methods have been used for various food crops including roots, tubers, cereals, and leaves. During the process the plant parts with cyanogens are generally disintegrated, leading to the release of HCN which is then volatilize in further processing techniques, such as roasting and drying [43].

2.6.1 Cassava

Boiling, steaming, baking or frying of whole fresh cassava roots is not very effective and usually results in HCN retention of 50 % or more. The reason for low efficacy of HCN removal is that the enzyme linamarase is inactivated with high temperature and cannot hydrolyse the heat-stable linamarin to glucose and acetone. In addition, the contact of cyanogens with linamarase is poor because the plant cells are mostly intact. To achieve significant reduction of HCN the plant cells need to be mechanically disrupted (by crushing, repeated pounding,

grating, or soaking in water for several days) following by fermentation and roasting processes. During fermentation of grated roots, linamarin is degraded to its cyanohydrin, which is quite stable at the acidic pH of the fermentation but decomposes to HCN and acetone during roasting.

Considering cassava flour, a 'wetting method' was developed to reduce content of HCN, and it is hoped to help prevent cyanide poisoning and konzo in African countries. The method is very simple; thin layer of wet cassava flour is kept in the shade for a few hours so the residual linamarase can degrade the residual cyanogens. Cassava leaves are usually processed by pounding followed by boiling, which lower the level of cyanogens up to 3% of the original amount. However, during these processes more than half of the proteins and water-soluble vitamins is lost. Therefore, milder methods have been proposed, e.g. pounding the leaves, followed by 2 h in the sun or 5 h in the shade, and finally three times washing with water. The residual content of cyanogens after these steps was 28, 12 and 1%, respectively [67].

2.6.2 Bamboo shoots

Cooking and boiling greatly reduces the level of cyanogens in bamboo shoots. Analysis showed that cyanogenic glycoside in the shoots of *D. hamiltonii* and *D. giganteus* were reduced by 68 % and 77 % percent respectively after 10 minutes boiling and up to 87% after 20 minutes boiling. Slicing and cooking bamboo shoot in boiling water for 15 minutes resulted in reduction of the content of cyanogens up to 91%. Another often used practice is soaking, which is quite effective in eliminating cyanogens particularly in those species with low content. The decrease in cyanogen depends on temperature and time of soaking as well as on soaking medium [4].

2.7 Methods of Quantification

Different methods for the qualitative, semi-quantitative or quantitative analysis of cyanogenic compounds are used [68]. Cyanogenic glycosides can be quantified either indirectly (by determining HCN released after hydrolysis) or directly (by determining the intact glycoside) [69]. Due to the medical importance of cyanide, most research data are reported for potential cyanide yields rather than the glycoside content itself [68].

2.7.1 Quantification of cyanogenic glycosides

Due to diverse chemical nature of cyanogenic glycosides, the extraction and analysis of individual compounds can be difficult. Ultrasound-assisted extraction (UAE) is a fast technique avoiding the hydrolysis of the cyanogenic glycosides. Pressure and cavitation generated by the ultrasonic waves cause the puncture of the plant cells, which facilitates the extraction of the cyanogenic glycosides. Ultrasonic probes are immersed in the sample and distribute the ultrasonic intensity directly to the liquid sample, enhancing the extraction yields and reducing the extraction time. Extracts containing cyanogenic glycosides are usually analysed by reverse phase HPLC with UV–VIS detectors, HPLC-MS, or tandem HPLC-DAD-MS [5]. These techniques can be used only for the known cyanogenic glycoside with standard materials, and it is difficult to estimate the total cyanogenic compounds in plant. Besides, tannins, flavonoids and chlorophyll in plant tissue have been shown to interfere with the liquid chromatographic determination of the cyanogenic glycosides [26]. Another difficulty of the method is the separation of the R and S epimers, for which reason some authors have proposed the use of chiral phases [5]. Except liquid chromatography-based techniques, GC-MS and ELISA have been used [67].

2.7.2 Quantification of total cyanide

Total cyanide is cyanide produced by complete hydrolysis of cyanogens and cyanohydrins present in food samples. Cyanogenic compounds can be hydrolysed either by acid catalysis or by enzymatic degradation. Food samples should be incubated with enzymes or diluted acid in sealed containers to ensure no leakage of released cyanide. The European Standard EN 16160 of 2012 (HPLC-based measurement) exists for quantification of total cyanide in food [67]. The most common methods are acid hydrolysis, picrate method, colorimetric method via König reaction and the chromatographic method. The colorimetric method includes extraction of cyanogenic compounds from the plant material, acid hydrolysis of cyanogenic glycoside, and the colour development and detection of cyanide. Colour development via König reaction detects except cyanide also thiocyanate and other plant secondary metabolites and thus makes the method less specific to cyanide. During the acid hydrolysis, HCN gas may leak, giving underestimated results. If intrinsic or extrinsic enzymatic hydrolysis is used, the enzyme might be inhibited by tannins from the plant tissue causing more underestimation [70], [71].

2.7.2.1 *Picrate method*

Picrate method is enzyme-based method for total cyanide detection based on reaction of picric acid with HCN that cause change of colour from yellow to red. In the first step of the procedure, HCN is liberated from crushed, disintegrated plant material via exogenous enzymes and then allowed to react directly with sodium picrate paper strips which are suspended above the solution. After certain period of time, the paper is removed and can be analysed qualitatively or quantitatively [71]. The reacted picric acid is qualitatively inspected using a colour chart **(**Figure 8) and relating the colour change of the paper to total amount of CN^- evolved. This simple and quick method serve as an effective method for quantifying total cyanides ranging between 1 and 100 μg. However, with increasing amount of cyanide is the differentiation between the colours more difficult [1]. In the quantitative way, the picric paper is after the reaction removed from the vial, put into a cuvette, and immersed in water for at least 30 minutes. The absorbance of the solution is then measured at 510 nm to calculate the cyanide content [72].

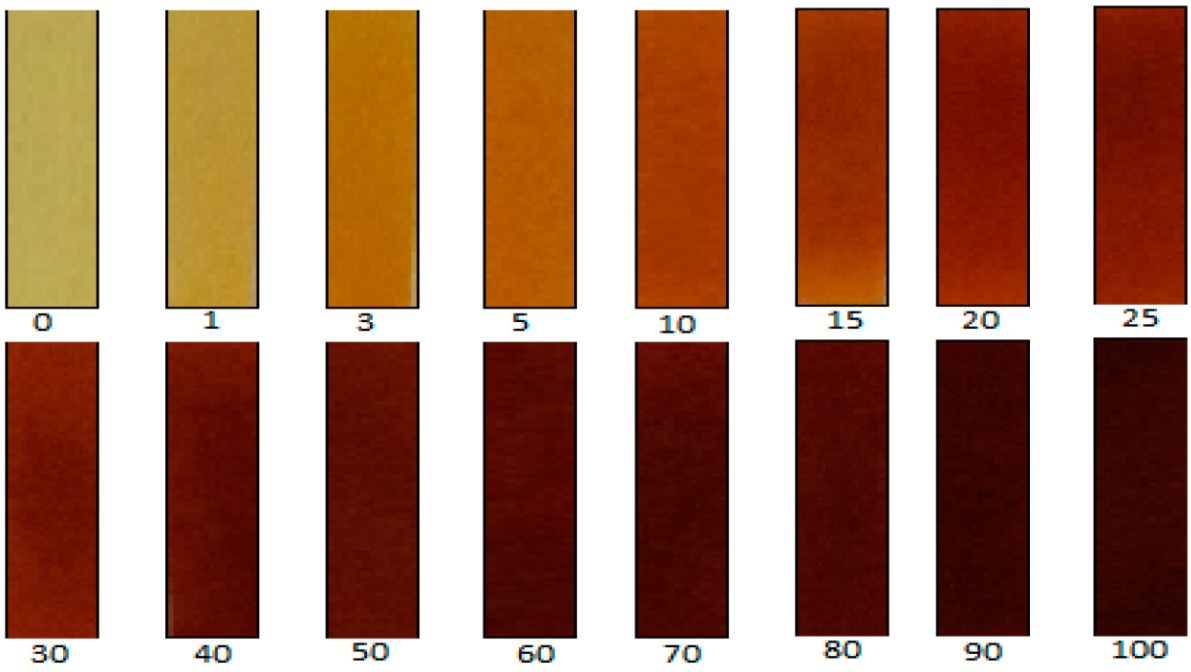


Figure 8: A picrate paper colour chart for qualitative analysis of total cyanides [39].

3 EXPERIMENTAL PART

3.1 Material

The plant material involved 46 elderberry genotypes. As shown in Table 3 and Table 4 five genotypes belonged to the species *Sambucus nigra* (two local genotypes belonging to *S. nigra* subsp. *nigra*, *S. nigra* var. *viridis*, *S. nigra* var. *laciniata*, and the cultivar *S. nigra* 'Black Beauty'), and the rest were interspecific hybrids. Each interspecific hybrid involved a self-incompatible genotype *S. javanica* (Chinese or Javanese elderberry) originating from the Island of Espiritu Santo, Vanuatu. The parental material of interspecific hybrids was considered as very heterozygous and therefore each offspring individual originating from the same cross represented a different genotype. This means that two or more hybrids with the same hybrid structure (e.g., (JA × NI) × BB) should be considered as genetically different. Except two C1 clones (JA × NI) × BB C1 and JA × CER No 3 C1 the plants originated directly from the seed. Majority of hybrids belonged to the third cycle of the recurrent selection program and were created at the University of Maribor, Faculty of Agriculture and Life Sciences at Hoče near Maribor, Slovenia. The sampled plants were three to four years old shrubs. Samples of berries, inflorescences, leaves, shoots, bark, and roots were taken from each plant. Shoots were sampled first, followed by other plant parts taken all at the same date. Since each genotype matured at different dates, plants were sampled over a range of dates. After sampling, the plant material was frozen in liquid nitrogen, freeze-dried, crushed into a fine powder, vacuum packed, and stored at −80 °C until analysed [73].

Table 3: Elderberry species and interspecific hybrids included in the investigation divided into groups based on their relatedness displayed with number of genotypes [73].

Material	Abbreviation	No.
Species		
Sambucus nigra	NI	2
S. nigra var. *viridis*	VIR	1
S. nigra var. *laciniata*	LAC	1
S. nigra ´Black Beauty´	BB	1
Interspecific hybrids exhibiting combination of traits of S. nigra and S. javanica		
(*S. javanica* × *S. nigra*) × (*S. javanica* × *S. nigra*)	(JA × NI) × (JA × NI)	1
(*S. javanica* × *S. nigra*) × *S. nigra* ´Black Beauty´C1	(JA × NI) × BB C1	5
(*S. javanica* × *S. nigra*) × *S. nigra* ´Black Beauty	(JA × NI) × BB	2
((*S. javanica* × *S. nigra*) × *S. nigra*) × ((*S. javanica* × *S. nigra*) × *S. nigra* ´Black Beauty´)	((JA × NI) × NI) × ((JA × NI) × BB)	6
((*S. javanica* × *S. nigra*) × *S nigra* ´Black Beauty´) × (*S. javanica* × (*S. javanica* × *S. nigra*))	((JA × NI) × BB) × (JA × (JA × NI))	1

Table 4: Cont.

Material	Abbreviation	No.
Interspecific hybrids exhibiting similarity to *S. ebulus*		
S. javanica × (*S. javanica* × *S. ebulus*)	JA × (JA × EB)	1
Interspecific hybrids exhibiting similarity to *S. racemosa*		
((*S. javanica* × *S. nigra*) × *S. racemosa*) × ((*S. javanica* × *S. nigra*) × *S. nigra* ´Black Beauty´)	((JA × NI) × RAC) × ((JA × NI) × BB)	2
(((*S. javanica* × *S. nigra*) × *S. nigra*) × *S. racemosa - miquelii*)	JA × (((JA × NI) × NI) × MIQ)	3
(*S javanica* × (*S. javanica* × *S. racemosa - miquelii*)) × ((*S. javanica* × *S. nigra*) × *S. nigra* ´Black Beauty´)	(JA × (JA × MIQ)) × ((JA × NI) × BB)	1
((*S. javanica* × *S. nigra*) × *S. nigra* ´Black Beauty´) × (*S. javanica* × *S. racemosa - miquelii*)	((JA × NI) × BB) × (JA × MIQ)	1
((S. javanica × S. nigra) × S. racemosa subsp. sibirica) × (S. javanica × S. nigra)	((JA × NI) × SIB) × (JA × NI)	1
Interspecific hybrids exhibiting similarity to *S. cerulea*		
S. javanica × *S. cerulea* No 3 C1	JA × CER No 3 C1	4
S. javanica × (*S. javanica* × *S. cerulea*)	JA × (JA × CER)	2
((*S. cerulea* × *S. nigra*) × *S. javanica*) × (*S. cerulea* × *S. nigra*)	((CER × NI) × JA) × (CER × NI)	1
Interspecific hybrids exhibiting combination of *S. cerulea* and *S. racemose*		
(*S. javanica* × *S. racemosa*) × (*S. cerulea* × *S. nigra*)	(JA × RAC) × (CER × NI)	1
((*S. javanica* × *S. nigra*) × *S. racemosa - miquelii*) × (*S. cerulea* × *S. racemosa - miquelii*)	((JA × NI) × MIQ) × (CER × MIQ)	1
((*S. javanica* × *S. cerulea*) × *S. racemosa - miquelii*) × ((*S. javanica* × *S. nigra*) × *S. cerulea*)	((JA × CER) × MIQ) × ((JA × NI) × CER)	2
((*S. javanica* × *S. nigra*) × *S. racemosa* subsp. *sibirica*) × *S. cerulea*	((JA × NI) × SIB) × CER	1
((*S. javanica* × *S. nigra*) × *S. racemosa* subsp. *sibirica*) × (*S. javanica* × *S. cerulea*) No 3	((JA × NI) × SIB) × (JA × CER) No 3	5

3.2 Laboratory Equipment and Facilities

- UV-VIS Spectrophotometer (Varian Cary 50 Bio)
- Analytical weight balance (Metler Toledo, Delta Range AT 261)
- Electronic pipette (Handy Step)
- Mili-Q water sytem (Elga)
- Centrifuge (Rotanta 460R, Hettich Zentrifugen)

3.3 Chemicals

- KH_2PO_4 (M_w= 136,09 g·mol^{-1}, 1112408, Kemika)
- Na_2HPO_4. $2H_2O$ (M_w= g·mol^{-1}, Lot = SZBE0760V, Sigma-Aldrich)
- Na_2CO_3 (M_w= g·mol^{-1}, CAS No 497-19-8, Riedel-de Haën)
- Picric acid (0.9-1.1%, Sigma-Aldrich)
- β-glucosidase (460 U/mL, Lot=151102a, Megazyme)
- Amygdalin (M_w= 427,43 g·mol$^{-1,}$ Lot=BCBZ8189, Sigma-Aldrich)
- Filter paper (MN 615, 150 mm)

3.4 Procedure

3.4.1 Preparation of Buffer

Phosphate buffer of pH 5 was prepared by combining 0.95 mL of solution A with 100 mL of solution B. Solution A was prepared by transferring 9.0786 od KH_2PO_4 into 1000 mL of Mili-Q water and solution B by transferring 11.876 of Na_2HPO_4. $2H_20$ into 1000 mL of Mili-Q water.

3.4.2 Preparation of enzyme solution

Enzyme solution was prepared by diluting certain amount of enzyme in Mili-Q water to reach final concentration of 3 mg/mL.

3.4.3 Preparation of picrate papers

Na_2CO_3 was weighed and transferred into a beaker, Mili-Q water and 1% solution of picric acid was added so the final solution contained 0.5 % of picric acid and 5 % of Na_2CO_3. Filter paper was cut into 2 cm wide strips, dipped into the solution, and allowed to dry. When dry, paper was cut into 1 cm wide squares, stapled with plastic strips and directly used or stored in refrigerator.

3.4.4 Quantification of total cyanide content in sample

All samples were prepared in triplicates. Plant material was weighed and transferred into the vial. Depending on the amount of sample different volumes of buffer and enzyme solution were added to the vial, so whole sample was moistened. Picrate paper squares attached to plastic strips were suspended above the sample, the vials were immediately closed and put into an oven at 40 °C for 20 hours. After 20 hours, vials were taken out and let to cool down. Picrate paper squares were removed from the plastic strips and transferred into Eppendorf tubes. 1 mL of Mili-Q water was added to each tube and samples were vortexed. After 30 minutes, papers were removed and tubes with coloured Mili-Q water were centrifuged for 15 minutes at 10 °C and

10 000 rpm to remove paper fibres that could interfere with following spectrophotometric measurement. The absorbance of the samples was measured at 510 nm.

3.4.5 Calibration curve

Amygdalin was used as a source of cyanide to develop calibration curve. A set of standard solutions of concentration 0.15, 0.25, 0.50, 1.0, 2.0, 4.0, and 6.0 mg CN^-/L were prepared from stock solution of amygdalin. Different volumes of stock solution were transferred into volumetric flask and diluted with Mili-Q water to give mentioned concentration. Volume of 0.5 mL of standard solution was then transferred into the vial, 0.5 mL of buffer and 0.05 mL of enzyme solution were added, and same procedure was followed as with samples. Blanks were prepared with adding 0.5 mL of buffer instead of amygdalin standard solution.

4 RESULTS AND DISCUSSION

The aim of this experiment was to determine total cyanide content in different elderberry plant parts. Simple picrate method was used for this experiment and forty-six elderberry genotypes were analysed. This chapter cover adaptation of picrate method for our experiment followed by observations considering calibration curve development and finally discusses the results with comparison to similar studies dealing with cyanide content in elderberry.

4.1 Preparation of picrate papers

Working range of the picrate paper method is dependent on amount of picric acid available in the vial that can react with released HCN from the sample. During the preparation the paper is dipped into picric acid solution and therefore the size and type of paper affect the final amount of picric acid. Bradbury and his team [72] compared picrate papers of the same size (3 x 1 cm) using chromatography paper Whatman No 1 and Whatman No 3MM which is two times thicker. Using No 1 paper the standard curve started to flatten already above 15 mg/L HCN, however Whatman No 3MM gave linear behaviour up to 40 mg/L HCN. Therefore, 3 x 1 cm large paper strips of Whatman No 3MM are the most used in picrate paper method. As seen in Figure 9, in our experiment, filter paper MN 615 was used, and papers were cut only into 1 x 1 cm squares to obtain lower sensitivity. It was important to ensure same conditions for all the paper squares: cut the squares accurately, dip them into picric acid solution for same amount of time, keep them stored in dark and cold and especially keep them dry when suspending in the vial.

Figure 9: Preparation of picrate papers and their final use in sample analysis.

4.2 Preliminary analysis of total cyanide content

Different parts of forty-six genotypes of elderberry tree were supposed to be analysed for total cyanide content. Firstly, pooled samples from more genotypes were observed to get preliminary results of total cyanide content in different parts (Table 5). The content of total cyanide decreased in order leaves > inflorescences > ripe berries > shoots > bark > roots. Cyanide content in root was found below the limit of detection (LOD) and low amounts were found in bark. Therefore, these two plant parts were excluded from further analysis.

Table 5: Total cyanide content found in different plant parts of elderberry.

Plant part	Weight of sample [mg]	HCN in mg/kg of fresh weight
Leaves	20	252.7 ± 9.6
Shoots	200	3.2 ± 0.1
Inflorescences	100	16.5 ± 0.9
Berries	300	9.6 ± 0.2
Roots	300	<LOD
Bark	300	0.7 ± 0.2

4.3 Effect of pH of the buffer

Enzymes β-glucosidases hydrolyse cyanogenic glycosides and produce benzaldehyde and HCN. Enzymes have their pH optimum around 5–6, therefore buffer of pH 5 is generally used in this method. However few studies reported higher cyanide yield obtained with buffer of pH 8. In study of Bradbury [72], higher total cyanide content was obtained in case of cassava and sorghum leaves and in the study of Appenteng [39], buffer of pH 8 was used for analysis of elderberry. The effect of different pH of the buffer was analysed in leaves, shoots, inflorescences and berries. Total cyanide contents when buffers of pH 5 and pH 8 were used are compared in Figure 10. With higher pH the total cyanide content in all elderberry parts decreased, thus, buffer of pH 5 was chosen for further analysis of all genotypes.

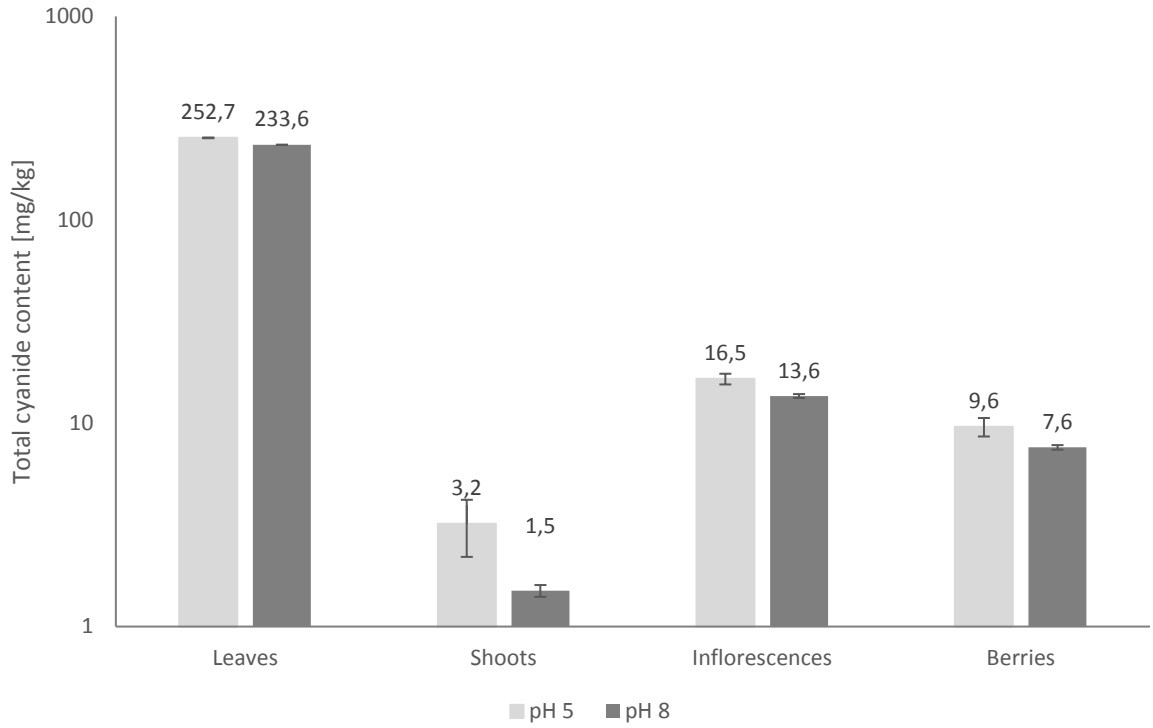


Figure 10: Total cyanide content determined in elderberry leaves, shoots, inflorescences and berries when buffers of pH 5 and pH 8 were used.

4.4 Calibration curve

As already mentioned, picrate paper method is based on reaction of picric acid with HCN which results in colour change of picrate paper. It serves both as qualitative and quantitative method. Qualitative method is used for rough estimation of total cyanide content in the sample by comparing colour of picrate papers from the sample with papers from the standard curve. In this experiment coloured papers were further analysed using spectrophotometry to obtain more accurate results. The calibration curve was prepared using nine different concentration of amygdalin standard solution ranging from 0.15 to 12 mg CN^-/L.

4.4.1 Qualitative analysis

Qualitative inspection of the picrate paper strips showed gradual colour change up to concentration of 6 CN^- mg/L. Difference among concentration 6, 9 and 12 CN^- mg/L is hardly noticeable due to saturation of picrate paper with HCN (Figure 11). Colour difference among blank (B1), concentrations of 0.15 CN^- mg/L and 0.25 CN^- mg/L are difficult to observe as well.

Figure 11: Colour change of picrate paper strips observed when samples were taken out from the oven after 20 hours.

4.4.2 Quantitative analysis

As colour development in Figure 11 suggested, with higher concentrations (9 CN^- mg/L and 12 CN^- mg/L) the standard curve flattened due to approaching saturation of the picrate paper with HCN (Figure 12). Since the flattening of the curve reduces the precision of the determination, the method was used only for concentrations of a maximum of 6 CN^- mg/L(Figure 13).

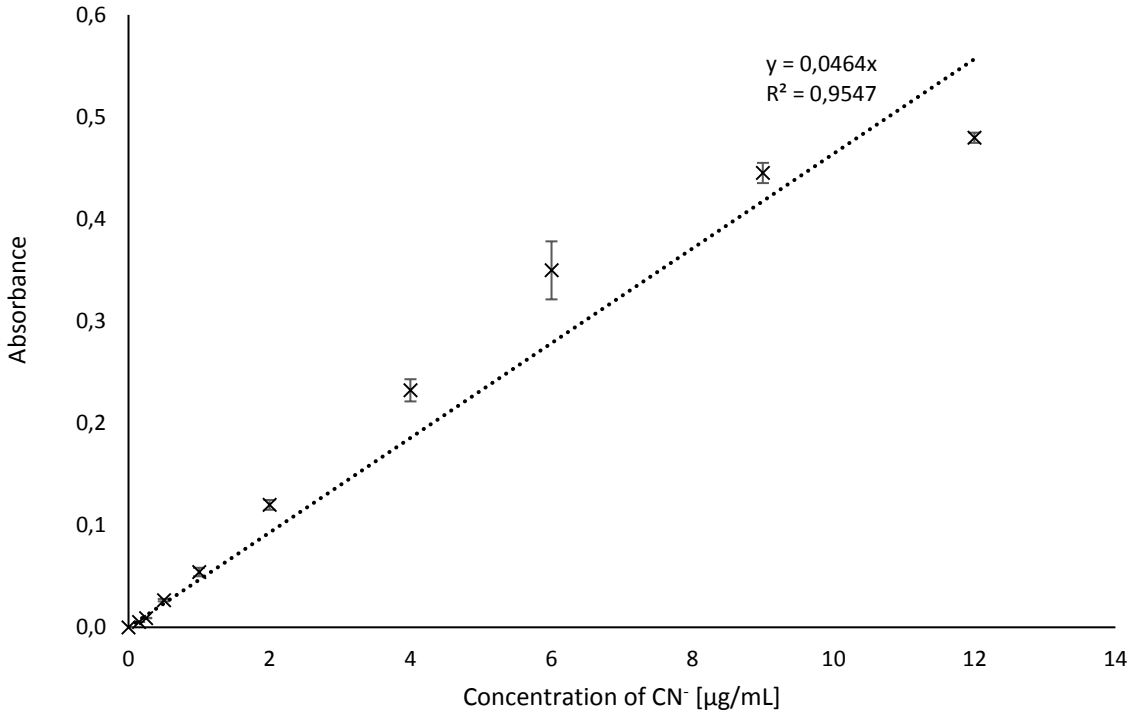


Figure 12: Standard curve of amygdalin displayed with linear regression and deviations. The curve starts to flatten at concentration of 9 CN^- mg/L as the picrate paper approaches saturation by CN^-.

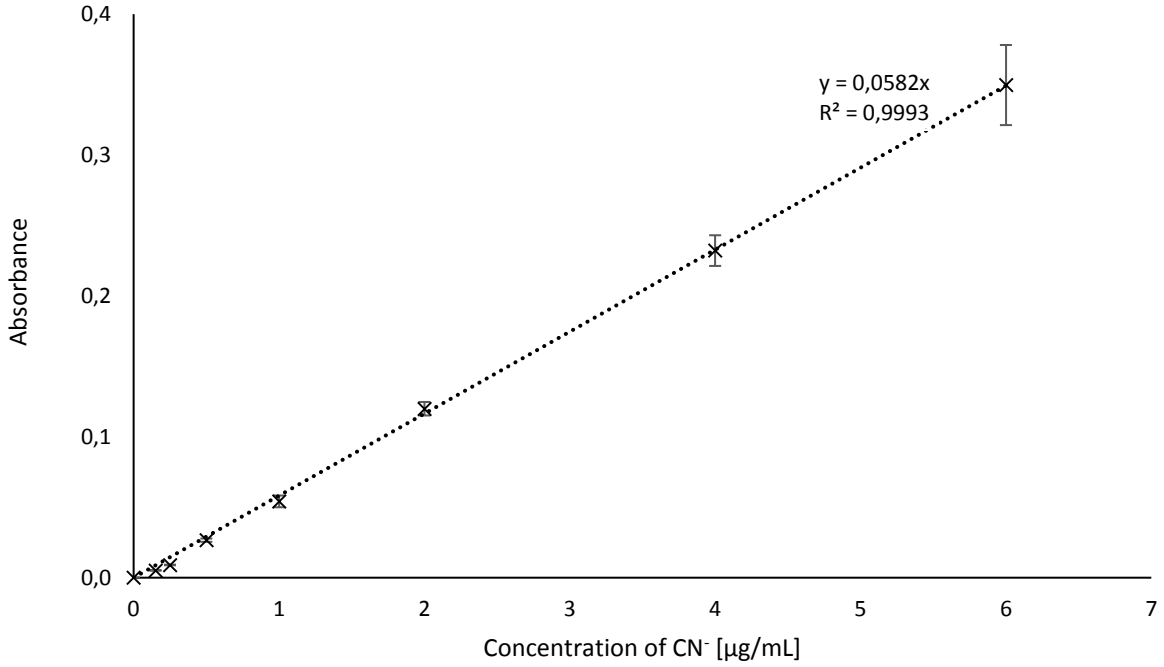


Figure 13: Standard curve of amygdalin solution displayed with linear regression and deviations. This standard curve was used in further analysis of the samples.

4.5 Total cyanide determination of total cyanide in elderberry genotypes

Plant material for the analysis included eighteen interspecific hybrids and four subspecies of *Sambucus nigra*, from which ten involved more than one genotype (2–6), giving forty-six genotypes in total. Plant parts included in the analysis were leaves, inflorescences, shoots and berries. All results are summed up in Table 6 and graphically presented in Figure 14. The highest total cyanide content was found in leaves varying widely among interspecific hybrids as well as among genotypes of one interspecific hybrid. Total cyanide levels in leaves of all forty-six genotypes ranged from undetectable amounts up to 1155.9 ± 44.8 CN^- mg/kg with mean value of 267.3 CN^- mg/kg. Cyanide levels in inflorescences were analysed in forty-five genotypes and ranged from non-detectable amounts up to of 53.9 ± 3.8 (if extreme value of 257.0 CN^- mg/kg is excluded) with mean value of 26.1 CN^- mg/kg. From forty-two genotypes tested for cyanide content in shoots, the cyanide levels were found below LOD in nineteen of them and maximum concentration reached 20.3 ± 1.3 CN^- mg/kg. Berries accumulated the lowest amount of total cyanide. From fourteen analysed genotypes cyanide was detected only in six, giving the highest value of 6.4 ± 0.0 CN^- mg/kg. The cyanide content decreased in a way: leaves > inflorescences > shoots > berries in all analysed genotypes apart from six. Analysis of each plant part is discussed in more detail in following chapters.

Table 6: Total cyanide content determined in different parts of elderberry genotypes divided by relatedness. NA – not analysed, <LOD – under the limit of detection.

Species/ interspecific hybrids	No. of Genotypes	Total cyanide content [ug/g]			
		Leaves	Inflorescences	Shoots	Berries
		Species of *S. nigra*			
NI	1	918.4 ± 26.9	43.3 ± 1.3	1.8 ± 0.1	2.0 ± 0.0
	2	812.3 ± 31.8	44.0 ± 2.6	4.1 ± 0.1	6.4 ± 0.0
VIR	1	275.7 ± 1.8	30.6 ± 0.5	1.5 ± 0.1	NA
LAC	1	956.8 ± 10.2	19.7 ± 0.9	6.2 ± 0.1	NA
BB	1	846.0 ± 17.5	12.5 ± 0.3	7.5 ± 0.7	NA
		Interspecific hybrids exhibiting combination of traits of *S. nigra* and *S. javanica*			
(JA × NI) × (JA × NI)	1	261.8 ± 16.9	20.5 ± 1.6	<LOD	<LOD
(JA ×NI) × BB C1	1	377.9 ± 4.1	28.8 ± 2.1	2.41 ± 0.1	<LOD
	2	293.6 ± 5.9	257.0 ± 3.9	12.9 ± 1.0	1.3 ± 0.1
	3	277.3 ± 0.8	19.2 ± 0.6	1.2 ± 0.2	NA
	4	252.5 ± 10.7	24.0 ± 2.8	0.4 ± 0.1	NA
	5	377.8 ± 14.1	39.2 ± 1.6	3.2 ± 0.1	<LOD
(JA × NI) × BB	1	587.4 ± 19.6	6.7 ± 0.7	1.0 ± 0.1	NA
	2	9.1 ± 0.2	10.1 ± 0.2	NA	NA
((JA × NI) × NI) × ((JA × NI) × BB)	1	1155.9 ± 44.8	30.4 ± 0.4	20.3 ± 1.3	0.5 ± 0.1
	2	89.1 ± 4.1	23.9 ± 1.1	<LOD	NA
	3	209.6 ± 3.7	31.1 ± 2.0	2.3 ± 0.0	NA
	4	796.9 ± 3.6	53.9 ± 3.8	4.3 ± 0.2	4.6 ± 0.0
	5	169.3 ± 3.1	24.5 ± 1.7	1.5 ± 0.1	2.9 ± 0.1
	6	250.6 ± 11.8	44.5 ± 2.7	3.6 ± 1.4	<LOD
((JA × NI) × BB) × (JA × (JA × NI))	1	93.7 ± 2.6	15.4 ± 1.4	0.5 ± 0.0	NA
		Interspecific hybrids exhibiting similarity to *S. ebulus*			
JA × (JA × EB)	1	7.6 ± 0.7	<LOD	<LOD	NA
		Interspecific hybrids exhibiting similarity to *S. racemosa*			
((JA × NI) × RAC) × ((JA × NI) × BB)	1	128.9 ± 12.3	4.3 ± 0.3	NA	NA
	2	94.6 ± 5.0	10.2 ± 0.5	<LOD	NA
JA × (((JA × NI) × NI) × MIQ)	1	20.9 ± 1.7	0.7 ± 0.1	NA	NA
	2	132.0 ± 2.7	9.2 ± 0.2	<LOD	NA
	3	281.3 ± 4.4	24.8 ± 1.3	1.6 ± 0.1	NA
(JA × (JA × MIQ)) × ((JA × NI) × BB)	1	502.1 ± 16.3	46.4 ± 0.3	1.7 ± 0.1	<LOD
((JA × NI) × BB) × (JA x MIQ)	1	82.1 ± 2.0	9.2 ± 0.0	<LOD	NA
((JA × NI) × SIB) × (JA × NI)	1	362.1 ± 9.8	1.3 ± 0.2	<LOD	NA

Table 7: Cont.

		Interspecific hybrids exhibiting similarity to *S. cerulea*			
JA × CER No 3 C1	1	2.4 ± 0.2	<LOD	<LOD	NA
	2	3.2 ± 0.6	<LOD	<LOD	NA
	3	6.5 ± 0.5	<LOD	<LOD	NA
	4	4.4 ± 0.2	<LOD	<LOD	NA
	5	7.1 ± 0.1	<LOD	<LOD	NA
JA × (JA × CER)	1	104.1 ± 2.0	29.3 ± 1.7	14.0 ± 0.1	<LOD
	2	<LOD	2.0 ± 0.2	<LOD	NA
((CER × NI) × JA) × (CER × NI)	1	32.4 ± 0.4	1.0 ± 0.0	NA	NA
		Interspecific hybrids exhibiting combination of *S. cerulea* and *S. racemosa*			
(JA × RAC) × (CER × NI)	1	124.3 ± 3.8	31.9 ± 0.8	1.5 ± 0.0	<LOD
((JA × NI) × MIQ) × (CER × MIQ)	1	9.3 ± 0.5	6.5 ± 0.9	<LOD	NA
((JA × CER) × MIQ × ((JA × NI) × CER)	1	260.8 ± 3.5	NA	7.7 ± 0.7	NA
	2	59.6 ± 2.0	16.6 ± 1.0	<LOD	NA
((JA × NI) × SIB) × CER	1	95.4 ± 4.0	2.7 ± 0.1	<LOD	NA
((JA × CER) × SIB) × (JA × CER) No 3	1	132.0 ± 4.7	2.0 ± 0.2	<LOD	NA
	2	27.6 ± 2.5	1.4 ± 0.1	<LOD	NA
	3	641.3 ± 29.6	23.2 ±1.5	0.81 ± 0.1	<LOD
	4	1.6 ± 0.0	<LOD	<LOD	NA
	5	160.4 ± 2.5	17.3 ± 0.5	2.2 ± 0.2	NA

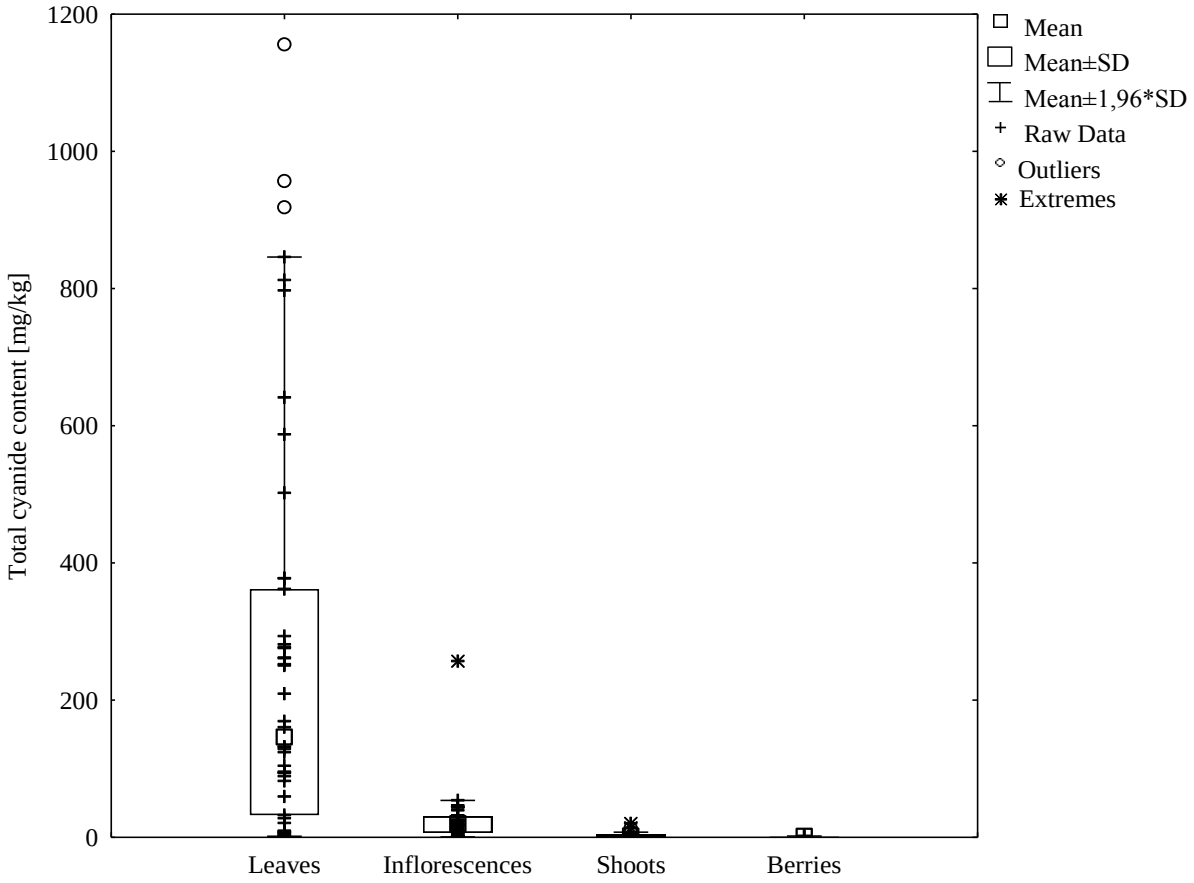


Figure 14. Box plot of total cyanide content found in elderberry leaves (46 genotypes), inflorescences (39 genotypes), shoots (25 genotypes), and berries (14 genotypes).

4.5.1 Leaves

Total cyanide contents in leaves obtained from the analysis of forty-six genotypes are displayed in Figure 15. Interspecific hybrid with the lowest amount of total cyanide is JA × CER No 3 C1. This hybrid comprises five genotypes and the total cyanide content in their leaves ranged from 2.4 CN^- mg/kg to 7.1 CN^- mg/kg. Low cyanide content (7.6 ± 0.7 CN^- mg/kg) was determined also in leaves of genotype JA × (JA × EB). No cyanide was detected in one genotype of interspecific hybrid of JA × (JA × CER), however another genotype of this hybrid contained 104.1 ± 2.0 CN^- mg/kg. The highest cyanide content (1155.9 ± 44.8 CN^- mg/kg) was found in a genotype of interspecific hybrid ((JA × NI) × NI) × ((JA × NI) × BB). This interspecific hybrid comprised six genotypes that varied widely in their total cyanide content (from 89.1 ± 4.1 8 CN^- mg/kg up to 1155.9 ± 44.8 CN^- mg/kg). Excluding specie VAR, high levels of cyanide were identified in all other species of *S. nigra* including LAC with 956.8 ± 10.2 CN^- mg/kg, NI with 918.4 ± 26.9 CN^- mg/kg and 812.3 ± 31.8 CN^- mg/kg and BB with 846.0 ± 17.5 CN^- mg/kg. Leaves accumulated the highest levels of cyanide compared to other plant parts in all genotypes except two; genotype of JA × (JA × CER) and ((JA × NI) × BB) exhibited more cyanide in inflorescences.

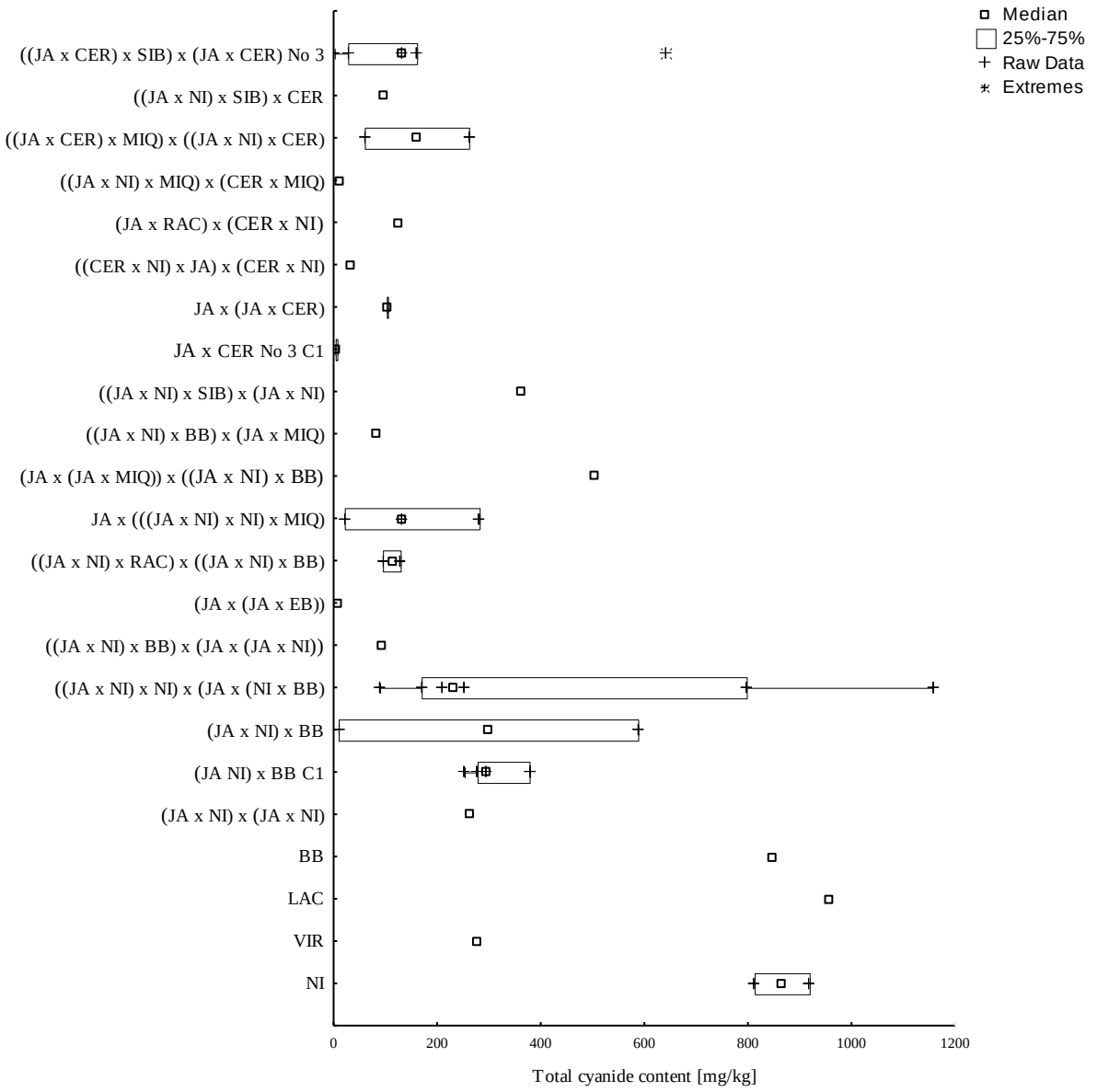


Figure 15: The cyanide content found in leaves of 46 elderberry genotypes.

4.5.1.1 ***Comparison with literature***

Several other studies analysed elderberry tree parts for their cyanide content. Accumulation of cyanide in plants has been linked to plant-herbivore interactions and adaptation to environmental conditions such as latitude, altitude, precipitation, temperature, light intensity, nutritional status, and many others. The plants from various studies were not grown in the same conditions and the cyanide content was analysed by different methods, therefore some differences among the results are expected. M. Senica [1] and her team analysed and compared total cyanide and phenolics content in leaves, inflorescences and berries of *S. nigra* (black elderberry), *S. ebulus* (dwarf elderberry) and *S. racemosa* (red elderberry) using HPLC-MS. Their results are compared with our values in Table 8. The highest content of cyanogenic glycosides was determined in plant parts of *S. nigra*, particularly in leaves, while in *S. ebulus* and *S. racemosa* the highest levels occurred in inflorescences. The cyanide content in all plant parts were substantially higher in *S. nigra* (reaching up to 1033.2 ± 25.8 CN^- mg/kg in leaves)

than in the remaining two species and the cyanide content in different plant parts of *S. nigra* followed trend leaves > inflorescences > berries. These findings are similar in our study, since the four genotypes of *S. nigra* species (excluding specie VIR) contained one of the highest cyanide levels (in range of 812.3–956.8 CN^- mg/kg). The two remaining species (*S. ebulus* and *S. racemosa*) were not involved in our analysis, however interspecific hybrids exhibiting traits of *S. ebulus* and *S. racemose* were included. Considering *S. ebulus*, one genotype of interspecific hybrid JA × (JA × EB) was analysed and exhibited similar cyanide content in leaves as in Senica's study (7.9 ± 0.7 CN^- mg/kg and 8.8 ± 0.3 CN^- mg/kg, respectively). Further, eight genotypes of five hybrids exhibiting similarity to *S. racemose* were analysed. Cyanide levels differed substantially among genotypes (20.9–502.1 CN^- mg/kg) and were significantly higher than in the investigation of Senica (1.1 ± 0.1 CN^- mg/kg). However, our results are from analysis of various interspecific hybrid not from pure specie *S. racemose*. This might imply that other traits used in breeding of interspecific hybrids influence resulting cyanide content. In our study the cyanide content decreases in a way: leaves > inflorescences > berries in majority of the genotypes, however in Senica's study this trend is followed only in *S. nigra*.

Table 8: Comparison of cyanide content in leaves from Senica's experiment and our analysis.

Comparison of cyanide content [mg/kg]			
S. nigra	Senica	*S.nigra*	1033.0 ± 25.8
	Our analysis	NI	918.4–812.0
		VIR	275.7 ± 1.8
		LAC	956.8 ± 10.2
		BB	846.0 ± 17.5
S. ebulus	Senica	*S. ebulus*	8.8 ± 0.3
	Our analysis	JA × (JA × EB)	7.9 ± 0.7
S. racemosa	Senica	*S. racemosa*	4.5 ± 0.3
	Our analysis	((JA × NI) × RAC) × ((JA × NI) × BB)	94.6 128.9
		JA × (((JA × NI) × NI) × MIQ)	20.9–281.3
		(JA × (JA × MIQ)) × ((JA × NI) × BB)	502.1 ± 16.3
		((JA × NI) × BB) × (JA x MIQ)	82.1 ± 2.0
		((JA × NI) × SIB) × (JA × NI)	362.1 ± 9.8

M. Senica and co-authors studied also the change in phenolics and cyanogenic glycosides content in trees of Black elderberry (*Sambucus nigra*) induced by different altitudes and locations. Phenolics and cyanogenic glycosides were determined in elderberry leaves, flowers, and berries collected at two different locations (continental and Mediterranean climate) and at four different altitudes (approximately 200, 400, 800 and 1000 m a.s.l.). Using HPLC-MS, sambunigrin, as the main cyanogenic glycoside, has been identified in all samples (Table 9). The content of sambunigrin in elderberry leaves was the highest at the highest altitude compared to the lowest at both locations ranging from 28.8 mg/kg to 75.4 mg/kg at location 1

and from 153.3 mg/kg to 209.6 mg/kg at location 2. The cyanide content decrease in the same pattern as in our experiment (leaves > inflorescences > berries). Significant differences in levels of sambunigrin have been determined between the two locations both for flowers and leaves [26].

Table 9: *Levels of sambunigrin found in elderberry leaves sampled at two different locations* [26].

Continental climate	**210 m**	**420 m**	**800 m**	**1048 m**
Sambunigrin [mg/kg]	28.8 ± 1.1	60.8 ± 6.3	31.0 ± 5.5	75.4 ± 9.7
Mediterranean climate	**209 m**	**450 m**	**858 m**	**1077 m**
Sambunigrin [mg/kg]	153.3 ± 3.2	27.6 ± 6.4	67.5 ± 8.2	209.6 ± 15.6

In study of Imenšek [73] same plants that are used in our experiment were previously investigated for content of oxalates in berries. It was found out that levels of oxalates in berries taken from trees originating from different seeds (e.g. ((JA × NI) × NI) × ((JA × NI) × BB)) and JA × (JA × CER)) varied more among genotypes of one hybrid than in clonally propagated plants (e.g. JA × CER No 3 C1 and (JA × NI) × BB C1). Similar observation was found in our analysis, since cyanide levels in genotypes of ((JA × NI) × NI) × ((JA × NI) × BB)) ranged between 89.1–1155.9 CN^- mg/kg and in JA × (JA × CER) between 0–104.1 CN^- mg/kg, whereas levels in clonally propagated plants of JA × CER No 3 C1 and (JA × NI) × BB C1, the levels ranged between 2.4–7.1 CN^- mg/kg and 252.0–377.8 CN^- mg/kg, respectively.

4.5.2 Inflorescences

The cyanide levels in inflorescences were analysed in forty-five genotypes. In all five genotypes of interspecific hybrid JA CER No 3 C1 cyanide content was below LOD as well as in genotype ((JA × CER) × SIB) × (JA × CER) No 3. Surprising result exhibit one genotype of (JA × NI) × BB C1 hybrid with its cyanide content of 257.0 ± 3.9 CN^- mg/kg, while remaining genotypes of this hybrid ranged in cyanide levels between 19.2–39.2 CN^- mg/kg. The content of cyanogenic glycosides is a response of individual plants to environmental factors and serve as protections against herbivores. Since all genotypes grew at same place, environmental conditions such as different weather or altitude can be neglected. Explanation for such high value might be that sampled inflorescences were attacked by herbivores and therefore synthesized more toxins for defence. The probability of mistake during the analysis is low since the samples were measured in triplicates and whole procedure was repeated twice. The contamination during sampling is not probable as well. If the value of 257.0 ± 3.9 CN^- mg/kg is excluded, the cyanide content in genotypes reached maximum of 53.9 ± 3.8 CN^- mg/kg and was found in a genotype of interspecific hybrid ((JA × NI) × NI) × ((JA × NI) × BB).

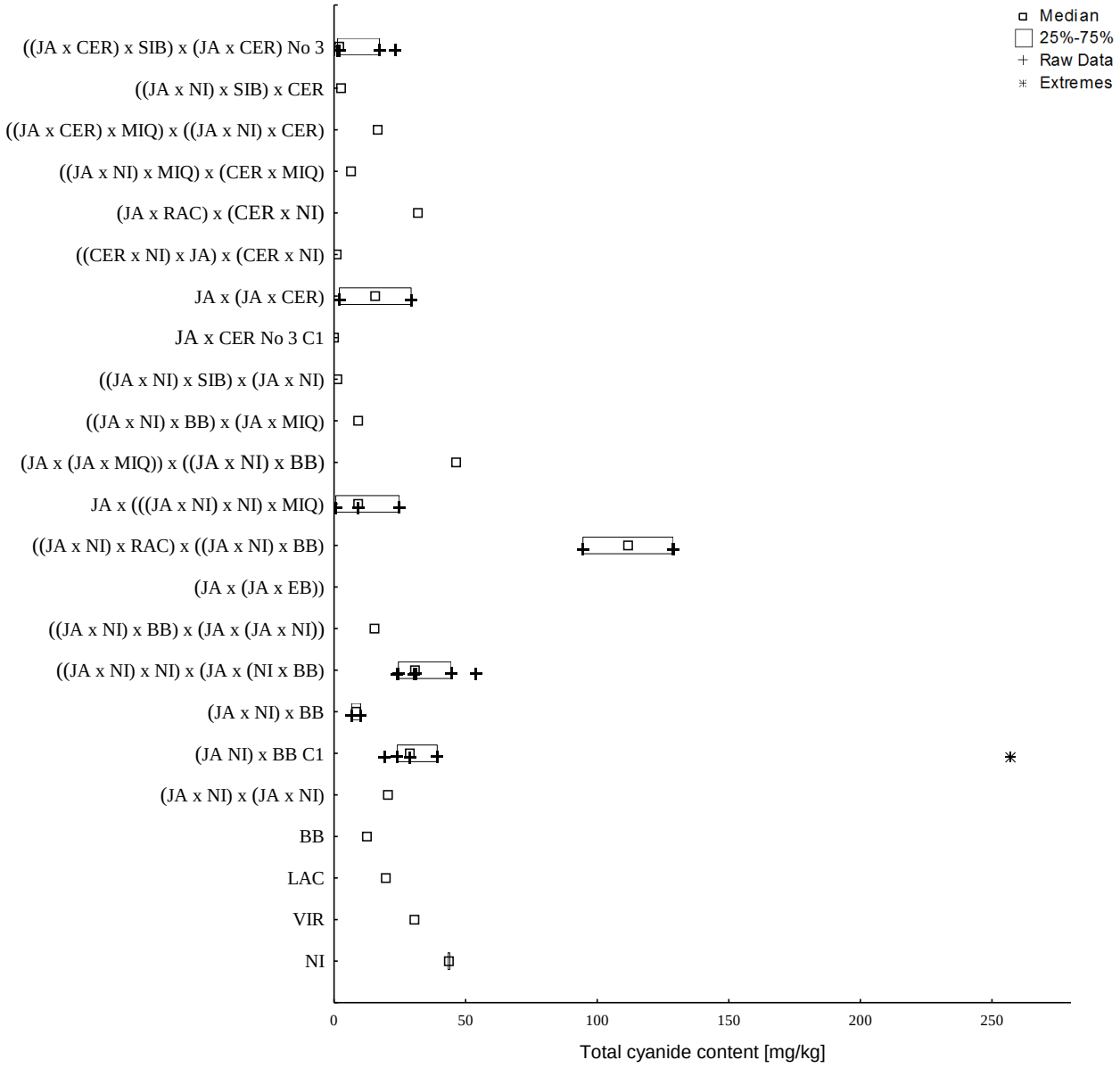


Figure 16: The cyanide content found in inflorescences. From forty-five analysed samples the cyanide was not detected in five genotypes.

4.5.2.1 ***Comparison with literature***

Total cyanide contents in leaves of *S. nigra* species were similar compared to Senica's results [1], however they extensively differ in inflorescences (Table 10). Cyanide levels of four species of *S. nigra* ranged between 12.5–44.0 CN^- mg/kg in our study, which is approximately ten times less compared to Senica's results (414.2 ± 15.6 CN^- mg/kg). Considering *S. racemose* cyanide content varied from 0.7 C to 46.4 CN^- mg/kg in various interspecific hybrids while Senica determined 4.45 ± 0.3 CN^- mg/kg in *S. racemose*. Cyanide content in genotype of JA × (JA × EB) was found below LOD however in Senica´s study inflorescences of *S. ebulus* accumulated 58.2 ± 3.6 CN^- mg/kg.

Table 10: Comparison of cyanide content in inflorescences from Senica's experiment and our analysis.

		Comparison of cyanide content	
S. nigra	Senica	*S.nigra*	414.2 ± 15.6
	Our analysis	NI	43.3 ± 1.3
		VIR	44.0 ± 2.6
		LAC	30.6 ± 0.5
		BB	19.7 ± 0.9
S. ebulus	Senica	*S. Ebulus*	58.2 ± 3.6
	Our analysis	JA × (JA × EB)	< LOD
S. racemosa	Senica	*S. racemosa*	4.5 ± 0.3
	Our analysis	((JA × NI) × RAC) × ((JA × NI) × BB)	4.3–10.2
		JA × (((JA × NI) × NI) × MIQ)	0.7–24.8
		(JA × (JA × MIQ)) × ((JA × NI) × BB)	46.4 ± 0.3
		((JA × NI) × BB) × (JA x MIQ)	9.2 ± 0.0
		((JA × NI) × SIB) × (JA × NI)	1.3 ± 0.2

In the second experiment of Senica's, the sambunigrin content in inflorescences ranged from 7.0 mg/kg to 18.8 mg/kg in location 1 and from 1.2 mg/g to 15.7 kg/g in location 2 (Table 11). Since only sambunigrin as a main cyanogenic glycoside was analysed, the total cyanide levels would be higher.

Table 11: Levels of sambunigrin found in elderberry inflorescences sampled at two different locations [26].

Continental climate	**210 m**	**420 m**	**800 m**	**1048 m**
Sambunigrin [μg/g]	7.0 ± 1.2	4.8 ± 1.1	11.0 ± 1.3	18.8 ± 0.8
Mediterranean climate	**209 m**	**450 m**	**858 m**	**1077 m**
Sambunigrin [μg/g]	1.2 ± 0.8	6.8 ± 2.0	6.1 ± 2.0	15.7 ± 1.7

Considering findings of Imenšek [73], in case of inflorescences the difference between cyanide levels in plants originated from seeds and clonally propagated plants was not that apparent. Cyanide levels in genotypes of ((JA × NI) × NI) × ((JA × NI) × BB)) ranged between 23.9–53.9 CN^- mg/kg and in genotype of JA × (JA × CER) between 2.0–29.3 CN^- mg/kg. Compare to clonally propagated genotypes of (JA × NI) × BB C1 ranging between 19.2–39.2 CN^- mg/kg (if extreme result of 257.0 ± 3.9 CN^- mg/kg is excluded). Levels in JA × CER No 3 C1 were in all genotypes found below LOD. On the other hand, two genotypes of NI which originate from seeds gave very close results (43.3 ± 1.3 CN^- mg/kg and 40.0 ± 2.6 CN^- mg/kg).

4.5.3 Shoots

Forty-two elderberry genotypes were tested for cyanide levels in shoots and in nineteen of them cyanide content was found below LOD. The highest total cyanide content of 20.3 ± 1.3 CN^- mg/kg was found in the same genotype of interspecific hybrid ((JA × NI) × NI) × ((JA × NI) × BB)) which had also the highest content in leaves from all genotypes. Other genotypes of this hybrid ranged from amount below LOD up to 4.3 ± 0.2 CN^- mg/kg. Levels in genotypes of (JA × NI) × BB C1 ranged between 0.4–12.9 CN^- mg/kg. There were not found any other studies investigating cyanide levels in shoots of elderberry that could be used to compare results.

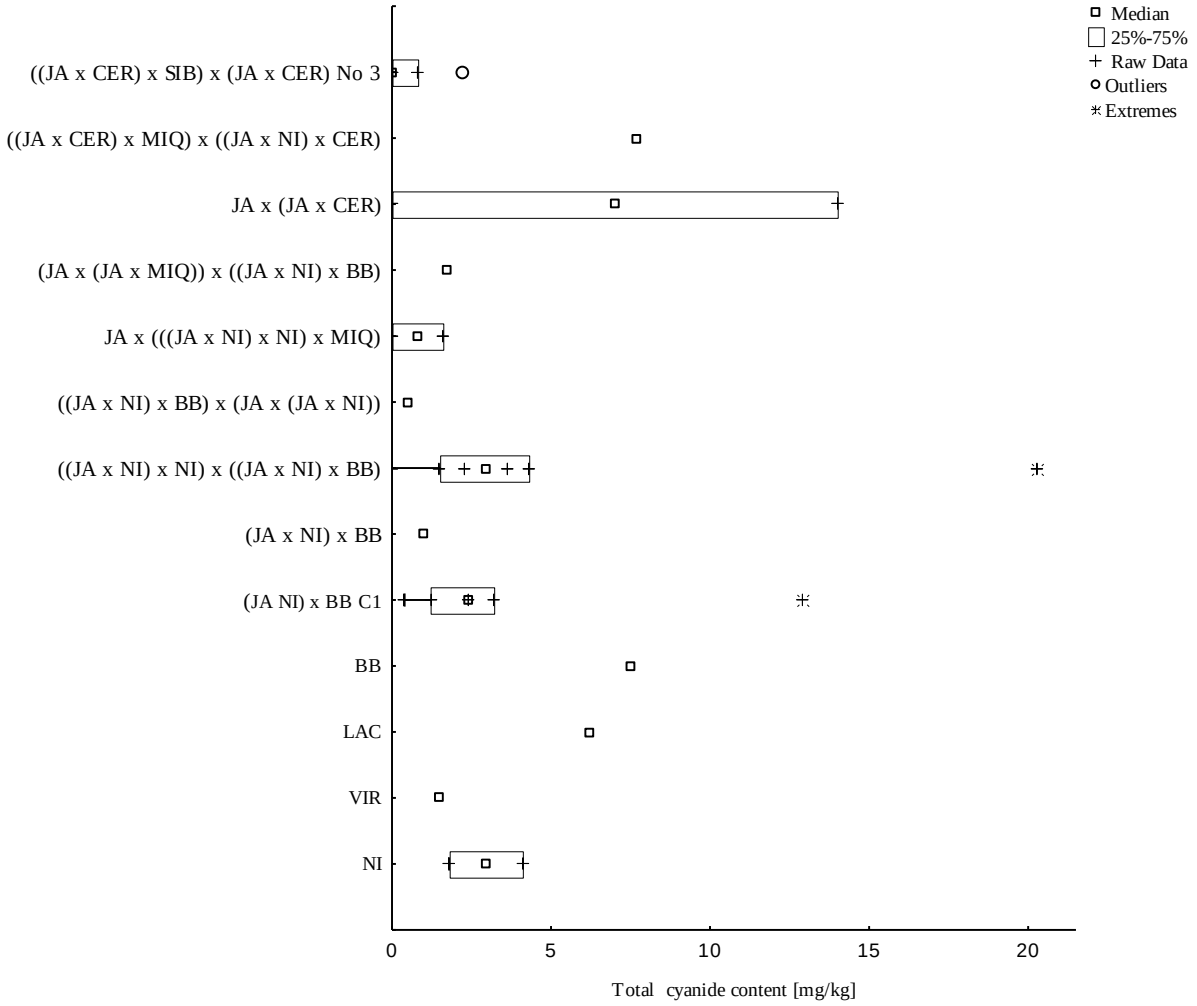


Figure 17: The cyanide content found in shoots. From forty-two elderberry genotypes the cyanide content was not detected in nineteen genotypes.

4.5.4 Berries

The cyanide content in elderberry berries was determined only in few genotypes of each group of species or interspecific hybrids giving fourteen genotypes in total. The number of genotypes was reduced due to high consumption of both the sample and enzyme and due to lower probability of cyanide detection. Very low amounts were detected in six genotypes (maximum of 6.4 ± 0.0 CN^- mg/kg) and no amounts were detected in eight genotypes. Cyanide content in berries of ten genotypes was lower than in shoots of the same genotype. However, in two genotypes of NI and two genotypes of ((JA × NI) × NI) × ((JA × NI) × BB) the content in berries was higher than in shoots.

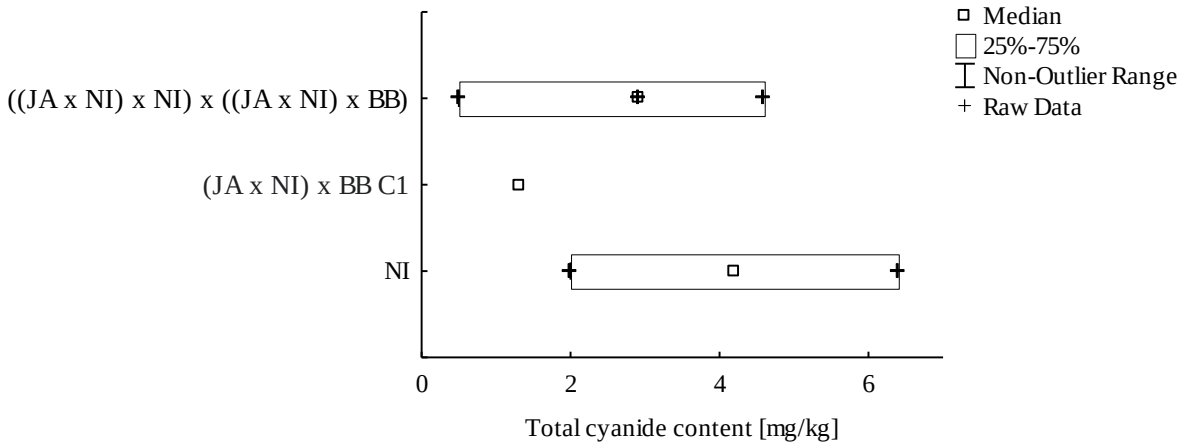


Figure 18: The cyanide content found in berries. From fourteen elderberry genotypes the cyanide content was detected in six genotypes.

4.5.4.1 *Comparison with literature*

Senica's results showed that 54.9 ± 1.3 CN^- mg/kg was accumulated in berries of *S. nigra,* 26.4 ± 1.4 CN^- mg/kg in berries of *S. ebulus* and 3.1 ± 0.2 CN^- mg/kg in berries of *S. racemose* (Table 12). In her other study the content in berries of *S. nigra* ranged between 0.08–0.35 CN^- mg/kg in continental climate and 0.11–0.77 CN^- mg/kg in Mediterranean climate (Table 13) and the trend of sambunigrin accumulation was not distinguishable among different altitudes in any location. In both studies berries were sampled when fully ripe.

Table 12: Comparison of cyanide content in berries from Senica's experiment and our analysis.

Comparison of cyanide content		
Senica	*S.nigra*	54.9 ± 1.3
Our analysis	NI	2.0–6.4
Senica	*S. racemosa*	3.1 ± 0.2
Our analysis	(JA × (JA × MIQ)) × ((JA × NI) × BB)	<LOD

Table 13: Levels of sambunigrin found in elderberry berries sampled at two different locations [26].

Continental climate	**210 m**	**420 m**	**800 m**	**1048 m**
Sambunigrin [μg/g]	0.24 ± 0.08	0.12 ± 0.02	0.08 ± 0.01	0.35 ± 0.03
Mediterranean climate	**209 m**	**450 m**	**858 m**	**1077 m**
Sambunigrin [μg/g]	0.11 ± 0.02	0.77 ± 0.08	0.36 ± 0.14	0.59 ± 0.12

The highest cyanide levels are expected to occur in unripe berries and decrease with ripening. M. K. Appenteng and his team [39] identified and quantified intact cyanogenic glycosides and the total cyanide content in different tissues of American Elderberry (AE) cultivars and in commercial AE juice. Samples of AE seeds, skin, pulp, stems, juice, and whole green, red, and ripe berries were analysed by picrate paper method. In a qualitative analysis a slight colour change of paper strips was observed for the stems and green berries. Following UV-VIS measurement showed that total cyanide content in analysed tissues increased in the order: whole ripe berries < whole red berries < juice < seeds < skin < pulp < whole green berries < stem, with highest average amounts found in the stems (37.43 ± 9.19 CN^- mg/kg) and whole green berries (25.6 ± 5.07 CN^- mg/kg). The total cyanide content in unripe green berries was more than double compared to ripe berries (10 CN^- mg/kg). In pressed juice samples, the concentration range measured was 0.29–2.36 μg/mL and in seeds the levels were 0.12–2.38 μg/g.

4.6 Evaluation of results regarding toxicity

Cyanide intake can cause acute toxicity in humans. The lethal dose is reported to be 0.5–3.5 mg/kg body weight, meaning consumption of 30–210 mg of cyanide for a person of 60 kg body weight. Table 14 displays amounts of different plant parts (mean values of results from all genotypes are used) that a 60 kg person would have to consume to reach lethal dose. Leaves pose the highest risk to human health however they are not used for consumption, similarly as shoots. Inflorescences and berries are the main plant parts used in food industry. The cyanide levels in these parts are quite low and therefore their consumption should not cause problems if intake of high doses is avoided. If higher quantities of inflorescences or berries and their products are consumed, the plant parts should be firstly crushed and then treated with heat by cooking or boiling to remove toxic HCN which evaporates.

Table 14: Lethal doses of different plant parts calculated from mean value of cyanide content in analysed samples.

Plant part	**Content of CN^- [mg/kg]**	**Lethal dose [kg]**
Leaves	267	0.1–0.8
Inflorescences	26	1.2–8.0
Shoots	12	2.5–17.5
Berries	3	10.0–70.0

5 CONCLUSION

The aim of this Master's thesis was to determine total cyanide levels in different parts of elderberry. The plant material included various hybrids, most of which belonged to the third cycle of recurrent selection program and were created at University of Maribor, Slovenia. The analysis included 4 subspecies of *Sambucus nigra* and 18 interspecific hybrids, each of them exhibiting 1 to 6 genotypes and giving 46 elderberry genotypes in total. To determine cyanide content picrate paper method and spectrophotometry were used. The calibration curve was prepared with amygdalin as a standard with working range between 0.15 and 6.0 μg CN^-/mL. The method was slightly optimized; size of the picrate paper strips was reduced from 3x1 cm to 1x1 cm squares to obtain lower sensitivity. Further, cyanide yields were compared when buffers of different pH were used and so was buffer of pH 5 chosen over buffer of pH 8 due to higher cyanide yields achieved.

Firstly, pooled samples of each plant part were tested for total cyanide content. The cyanide levels decreased in order leaves > inflorescences > berries > shoots > bark > roots. Since the levels in bark were very low and in roots below LOD, these two parts were excluded from further investigation of each genotype. From all plant parts the highest cyanide levels were found in leaves ranging from undetectable amounts up to 1155.9 ± 44.8 CN^- mg/kg. The highest amounts were accumulated in genotypes of interspecific hybrid ((JA × NI) × NI) × ((JA × NI) × BB) and subspecies of *S. nigra* NI, LAC, and BB while the lowest ones were found in interspecific hybrid JA × CER No 3 C1. Cyanide levels in inflorescences ranged from non-detectable amounts up to of 53.9 ± 3.8 (if extreme value of 257.0 ± 3.9 CN^- mg/kg is excluded). Same as in leaves, the highest levels were found in genotypes of ((JA × NI) × NI) × ((JA × NI) × BB) followed by (JA × (JA × MIQ)) × ((JA × NI) × BB) and specie of *S. nigra* NI. In all genotypes of interspecific hybrid JA × CER No 3 C1 the cyanide was not detected. Furthermore, cyanide content in shoots was not detected in 19 genotypes and the maximum was found to be 20.3 ± 1.3 CN^- mg/kg in one genotype of interspecific hybrid ((JA × NI) × NI) × ((JA × NI) × BB), though cyanide content in other genotypes of this hybrid were substantially lower. Considering berries, only 14 genotypes of elderberry were tested and in 8 of them amounts of cyanide were below LOD. The highest value of cyanide content was found in berries of subspecies *S. nigra* NI and reached 6.4 ± 0.0 CN^- mg/kg. Cyanide content decreased in order leaves > inflorescences > shoots > berries in all genotypes except six. Two genotypes exhibited higher cyanide yield in leaves than in inflorescences and in four genotypes berries accumulated higher levels than shoots.

Our findings were compared with different investigations. Considering leaves, our results were mostly in accordance with results of other studies, however for inflorescences and berries they differed. Since the cyanide levels are influenced by many factors such as weather, altitude, maturity stage of sampled plant part etc., it is natural that there are differences among our findings. There was not found any study investigating levels of cyanide in shoots.

Cyanide lethal dose is reported to be 0.5–3.5 mg/kg which would for 60 kg person mean approximate consumption of 0.1–0.8 kg of leaves, 1.1–8.1 kg of inflorescences, 2.5–17.5 kg of shoots and 10.0–70.0 kg of berries of elderberry (calculated from mean value of cyanide content in all genotypes for each plant part). Leaves possess the highest danger from all elderberry parts however they are not used for consumption. All the other elderberry parts should not cause

danger to consumers if not taken in very high doses. The plant parts could be also processed (crushing, boiling, cooking) before consumption to lower the risk to a minimum.

Due to high variability in cyanide levels among genotypes of same interspecific hybrid and due to low numbers of genotypes of one hybrid or species, it is not possible to give accurate analysis of hybrids and suggest the most suitable or unsuitable one for human consumption. However, it can be estimated that the interspecific hybrid ((JA × NI) × NI) × ((JA × NI) × BB) exhibits higher levels of cyanide and its plant parts should be treated more carefully before consumption. On the opposite JA × CER No 3 C1 and JA × (JA × EB) accumulated very low doses of cyanide and can be recommended for further breeding processes and use. However, there are many other important variables for choosing the most suitable elderberry species or interspecific hybrid, such as content of other bioactive compounds, mineral content, yield etc.

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7 LIST OF ABBREVIATIONS

AE	American Elderberry
BCE	Before Christian Era
GC-MS	Gas Chromatography - Mass Spectrometry
HCN	Hydrogen Cyanide
HPLC-DAD-MS	High-performance Liquid Chromatography Coupled with Diode-array Detection and Tandem Mass Spectrometry
HPLC-MS	High Performance Liquid Chromatography-Tandem Mass Spectrometry
EFSA	The European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
LOD	Level of Detection
rpm	Revolutions per Minute
UV-VIS	UltraViolet-Visible Spectroscopy

8 ATTACHMENTS

Table 15: Absorbances of standard solutions for calibration curve.

Concentration CN^- [mg/L]	A_1	A_2	A_3	A_4	$A_{average}$	Deviation
0.15	0.0052	0.0052	NA	NA	0.0052	0.0000
0.25	0.0089	0.0092	NA	NA	0.0091	0.0002
0.5	0.0249	0.0257	0.0230	0.0241	0.0244	0.0010
1	0.0528	0.0504	0.0505	0.0526	0.0516	0.0011
2	0.1172	0.1146	0.1145	0.1189	0.1163	0.0018
4	0.2267	0.2289	0.2453	0.2412	0.2355	0.0079
6	0.3496	0.3378	0.3350	0.3639	0.3466	0.0124
9	0.4553	0.4303	0.4498	0.4534	0.4451	0.0099
12	0.4746	0.4846	NA	NA	0.4796	0.0050