Agricultura 19: No 1: 43-50(2022)
https://doi.org/10.18690/agricultura.19.1.43-50.2022

University of Maribor Press

Cyanide Contents in Pits of Cherries, Gages and Plums Using a Modified Sensitive Picrate Method

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ABSTRACT

Cyanogenic glycosides are present in at least 2500 taxonomic groups of different plant families, including many economically important fruit species. They are most concentrated in the seeds of fruits and may pose a risk to human health due to the release of cyanide. In the present study, we compared the cyanide content in the pits of cherries (*Prunus avium* L.), plums (*Prunus domestica* L.), and gages (*Prunus domestica* subsp. *italica* (Borkh.) Gams) using the picrate paper method. The lowest cyanide content (19.6 µg/g) was found in a cherry accession (R x L 98), and the highest (310 µg/g) in a gage accession (Gage 6319). Considerable variation in cyanide content was found within varieties of a fruit tree species, reaching a coefficient of variation of 56.9% for cherries, 54.4% for plums, and 30.5% for gages. Looking at the overall medians, the results show that the cyanide content in the pits of gages was significantly higher than the cyanide content in the pits of plums and cherries.

Key words: cyanide, pits, cherries, gages, plums

INTRODUCTION

Cyanogenic glycosides (CGs) are organic compounds belonging to the group of plant secondary metabolites. Chemically, they are defined as α-hydroxynitrile glycosides to which the sugar moiety, usually glucose, is attached. At least 60 different CGs are found in more than 2500 taxonomic groups of the different plant families. The most studied are linamarin, amygdalin, prunasin, dhurrin, and lotaustralin which are found in greater amounts in agriculturally grown plants of economic interest (Vetter, 2000). CGs become toxic to humans when they are converted to hydrocyanic acid (HCN), during an enzymatic degradation process. Hydrolysis is initiated by mechanical damage to the cells such as grinding, rubbing, chewing, etc. HCN is acutely toxic to humans, with a predicted lethal dose of 0.5 to 3.5 mg/kg body weight (Speijers, 1993; Balhorn et al., 2009). Recently, EFSA (2016) reported a much lower acute toxic reference dose of 20 µg/kg body weight.

Specific CGs in certain plant materials are usually determined by liquid chromatography coupled with mass spectrometry (LC-MS) (Gómez et al., 1998; Badr and Tawfik, 2010; Lee et al., 2013; Arrazola et al, 2013). Santos et al. (2014) reported that amygdalin and prunasin in extracts of dried leaves can be quantified by nuclear magnetic resonance (NMR) spectroscopy. Determination of total cyanide content in plant materials requires cleavage of cyanogenic glycosides with acid or hydrolytic enzymes to release HCN (Haque and Bradbury, 2002; Drochioiu et al., 2008), which is then usually determined by the simple picrate paper method (Egan et al.,1998; Haque and Bradbury, 2002; Bradbury, 2009).

The function of cyanides in plant leaves and tissues is attributed to defense against herbivores and pests (Ballhorn, 2009). In contrast, the functions of CGs in seeds are less well studied and understood. Cyanogenic compounds are thought to be transferred from seed to seedling for defense and to function as nitrogen stores or germination inhibitors. Certain species in the rose family (Rosaceae) are known to

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contain higher concentrations of CGs. These include almonds (*Prunus Dulcis* Mill. D. A. Webb), apricots (*Prunus armeniaca* L.), peaches (*Prunus persica* L.), plums (*Prunus domestica* L.), cherries (*Prunus avium* L.), gages (*Prunus domestica* subsp. *italica* (Borkh.) Gams) etc. Cyanide content in apricot and peach kernels varied from 50 to about 4000 µg/g and from 400 to 2600 µg/g, respectively (Ballhorn, 2011). Black cherry (*Prunus serotina* Ehrh.) and cherry kernels contain amygdalin, neoamygdalin, and prunasin, with amygdalin present in higher amounts. Expressed as cyanide content, levels varied from 118 µg/g in 'Grace Star' to 297 µg/g in 'Black Star' (Senica et al., 2017), while lower levels (2.90 µg/g) were reported for black cherry (*Prunus serotina* Ehrh.) kernels (Brozdowski et al., 2021). The content varies greatly depending on fruit species, variety and many other factors (Ballhorn, 2011; Brozdowski et al., 2021).

Many cases of poisoning are reported due to consumption of apricot kernels. Cyanide levels in raw apricot kernels range from 0.5 to 3800 µg/g. An average kernel weight is about 0.5 g. Considering the highest cyanide content, an acute reference dose could be exceeded in children by eating one small apricot kernel and in adults by eating more than three small kernels or less than half of one large kernel (EFSA, 2016). According to the report by Senica et al. (2017), caution should also be considered when consuming the kernels of other fruits, especially plum, cherry, and peach kernels and seeds.

Plums, apricots, cherries and some other stone fruits are used for the production of liqueurs and alcoholic beverages and for homemade tinctures. The transfer of CGs from apricot and cherry pits into liqueurs was studied by Senica et al. (2016), who reported a much lower content in the liqueurs compared to the content in the pits and kernels. The concentration of CGs in liqueurs depended mainly on the original content in whole fruits. HCN was also found in canned fruit due to enzymatic hydrolysis of CGs during preservation, but the levels found were low (Voldřich and Kyzlink, 1992). Products such as spirits, liqueurs, and canned fruits may therefore still pose a potential risk to consumer health.

The objective of our study was to determine the content of total cyanide in the pits of plums (*Prunus domestica* L.), gages (*Prunus domestica* subsp. *italica* (Borkh.) Gams), and cherries (*Prunus avium* L.). Based on the results obtained, we could possibly make recommendations for the use of certain cultivars or varieties in processing operations that do not remove the pits of the fruit.

MATERIALS AND METHODS

Sample collection and preparation

Thirty cherry accessions, 20 gages accessions, and 20 plum accessions were included in the study. The trees are grown on the field of Plant Gene Bank of the University of Maribor, Faculty of Agriculture and Life Sciences in Hoče near Maribor and were selected using a random number generator. All cherry samples are crosses between two known cherry cultivars, *Prunus avium* 'Regina' and *Prunus avium* 'Lapins'. Plums were sorted into three groups according to their origin: primitive genotypes, planned crosses, and cultivated genotypes, and gages into two groups: primitive genotypes and planned crosses (Table 1).

Thirty fully mature fruits were randomly selected per tree. They were sampled from different parts of the canopy to reduce the possible influence of fruit position on the tree on the content of CGs. The fruits were harvested from June to August in 2020.

After harvest, the pits were removed from the pulp, washed with water, and dried at room temperature for 24 hours. The pits were then stored in airtight bags at -20 °C until further sample preparation. The next steps included cracking the pits with nutcracker tongs to expose the kernels, followed by freeze-drying (Christ Alpha 1–2 LD, Vacuumbrand GMBH, Germany). The pits were then ground to a relatively fine, homogeneous powder using a Tefal the Real Grinder GT110838. To avoid a potentially large heat input from grinding, the sample size was approximately 10 g and the grinding time was limited to 40 s. The samples were then vacuum sealed and stored at -80 °C until analysis.

Determination of total cyanide

The sensitive picrate method of Bradbury (2009), modified by Desser (2015), was used to determine total cyanide content in gage, plum, and cherry pits. Picrate papers were prepared by cutting filter papers (Machery-Nagel, MN 615) into 2 cm x 10 cm strips that were immersed in a 0.5% aqueous picric acid solution containing 5% sodium carbonate (Na2CO3) until completely saturated. They were then dried at room temperature and cut into 1 cm x 1 cm squares.

Samples (30 to 100 mg) of the freeze-dried, finely ground pits were weighed into headspace screw top vials. Then, 0.5 mL of phosphate buffer with a pH of 5.0 and 50 µl of β-glucosidase enzyme solution (2 mg/mL) (Thermotoga maritima with an activity of 73.8 U/mg protein) were added. Immediately after the addition of the enzyme, the picrate paper was also added to the vial so that it was suspended above the sample solution by tightly sealing the glass vial with a portion of the plastic strip remaining on the outside. The vials were placed in an incubator at 40 °C for at least 20 hours, and then cooled to room temperature. The picrate papers were detached from the plastic strips and transferred to 1.5 mL centrifuge tubes. Afterwards, 1 mL of Milli-Q water was added to elute the color from the papers. The solutions were shaken and centrifuged (15 min at 10 °C and 10.000 rpm). The absorbance of the solutions was measured at 510 nm on the UV/Vis spectrophotometer (Varian Cary 50 Bio). In parallel with the sample preparation, a blank sample and a calibration curve were prepared using amygdalin as a standard. The calibration curve was prepared in the range of cyanide content from 0.5 to 9 µg/ml. Each sample was analyzed in three replicates.

Statistical analysis

Statistical analysis was performed using the programme IBM SPSS Statistics 28. For each fruit variety we calculated the mean ($\bar{x}$), standard deviation (SD), minimum value

Table 1: Cherry, plum and gage accessions included in the study

Fruit species	Origin	Plant ID	
Cherry (*Prunus avium* L.)	*P. avium* 'Regina' x *P. avium* 'Lapins'	R x L 14	R x L 66
		R x L 53	R x L 43
		R x L 30	R x L 26
		R x L 101	R x L 32
		R x L 19	R x L 111
		R x L 105	R x L 49
		R x L 83	R x L 104
		R x L 98	R x L 123
		R x L 120	R x L 17
		R x L 34	R x L 94
		R x L 39	R x L 79
		R x L 78	R x L 45
		R x L 103	R x L 73
		R x L 97	R x L 29
		R x L 118	R x L 11
Plum (*Prunus domestica* L.)	Primitive genotype	Yellow plum	Local genotype No 9
		Red plum	Laška plum 3
		Laška plum 1	Laška plum 2
		Very old genotype	Natural hybrid Vrbanska
		Private collection plum No 1	
	Planned cross	3576 plum	
		I.A. Early dark plum	
		Small plum	
	Cultivated genotype	'Jojo'	'Hanita'
		Apriplum	'Ruth Gerstetter'
		'Elena'	'Elena' 2
		'Empress'	'Ersinger'
Gage (*Prunus domestica* subsp. *italica* (Borkh.) Gams)	Primitive genotype	Gage No 19	Gage No 27
		Gage No 17	Gage 6357
		Reddish yellow gage	Gage No 1
		Purple gage	Gage No 2
		Gage K22	Gage No 12
		Small-fruiting gage No 2	Gage 6403
		Gage 6319	Gage B6
		Gage B4	Gage No 16
		Gage 6378	Gage K-9
	Planned cross	I.A. 3 Gage	
		A.I. 8 Red-leaved gage	

(min), maximum value (max), median (me), first quartile (q_1), and third quartile (q_3). The nonparametric equivalent of an ANOVA, called the Kruskal-Wallis test, was conducted, because the assumption of homogeneity of variance and normality was violated. The results of this test indicated that there was a difference between the medians of at least one pair of groups, so we proceeded with the Dunn-Bonferroni post-hoc test.

RESULTS AND DISCUSSION

Total cyanide content in pits of cherries, plums and gages

The total cyanide content in the cherry pits examined in our study varied from 19.63 µg/g in the R x L 98 accession to 167 µg/g in the R x L 111 accession (Table 2). Cyanide ions were released from samples during enzymatic hydrolysis of amygdalin, prunasin, and neoamygdalin. Senica et al.

Table 2: Cyanide content in the pits of cherries, plums and gages. Results are averages of three measurements reported on a dry weight basis

Fruit species	Origin	Plant ID	Average cyanide content ± SD (μg/g)
Cherry (*Prunus avium* L.)	*P. avium* 'Regina' x *P. avium* 'Lapins'	R x L 14	36.9 ± 1.5
		R x L 53	27.9 ± 1.2
		R x L 30	127 ± 3
		R x L 101	107 ± 10
		R x L 19	99.1 ± 4.1
		R x L 105	25.2 ± 2.1
		R x L 83	28.3 ± 0.1
		R x L 98	19.6 ± 1.3
		R x L 120	20.5 ± 0.8
		R x L 34	66.5 ± 4.1
		R x L 39	140 ± 2
		R x L 78	83.1 ± 2.2
		R x L 103	58.2 ± 1.6
		R x L 97	40.2 ± 3.6
		R x L 118	54.3 ± 4.8
		R x L 66	59.8 ± 3.1
		R x L 43	62.2 ± 1.1
		R x L 26	105 ± 10
		R x L 32	77.9 ± 0.9
		R x L 111	167 ± 1
		R x L 49	48.2 ± 0.3
		R x L 104	35.0 ± 2.8
		R x L 123	72.8 ± 4.6
		R x L 17	26.1 ± 2.4
		R x L 94	27.0 ± 1.4
		R x L 79	69.7 ± 2.5
		R x L 45	100 ± 1
		R x L 73	119 ± 1
		R x L 29	54.0 ± 0.8
		R x L 11	93.0 ± 4.3
Plum (*Prunus domestica* L.)	Primitive genotype	Yellow plum	119 ± 1
		Red plum	173 ± 2
		Laška plum 1	165 ± 1
		Very old genotype	175 ± 4
		Local genotype No 9	74.9 ± 1.0
		Laška plum 3	50.4 ± 0.6
		Laška plum 2	76.1 ± 7
		Natural hybrid Vrbanska	182 ± 2
		Private collection plum No 1	185 ± 2
	Planned cross	3576 plum	253 ± 9
		I.A. Early dark plum	131 ± 1
		Small plum	35.6 ± 1
	Cultivated genotype	'Elena' 2	58.1 ± 1.1
		'Elena'	61.5 ± 0.9
		'Jojo'	140 ± 2
		Apriplum	169 ± 1
		'Empress'	116 ± 2
		'Hanita'	39.6 ± 0.8
		'Ruth Gerstetter'	37.1 ± 0.7
		'Ersinger'	70.4 ± 1.2

Gage (*Prunus domestica* subsp. *italica* (Borkh.) Gams)	Primitive genotype	Gage No 27	289 ± 15
		Gage 6357	177 ± 6
		Gage No 1	246 ± 9
		Gage No 2	190 ± 7
		Gage No 12	275 ± 21
		Gage 6403	255 ± 18
		Gage B6	293 ± 21
		Gage No 16	238 ± 8
		Gage K-9	124 ± 1
		Gage No 19	281 ± 18
		Gage No 17	270 ± 10
		Reddish yellow gage	276 ± 11
		Purple gage	124 ± 4
		Gage K22	159 ± 7
		Small-fruiting gage No 2	300 ± 12
		Gage 6319	310 ± 23
		Gage B4	226 ± 17
		Gage 6378	274 ± 25
	Planned cross	A.I. 3 Gage	58.6 ± 0.7
		A.I. 8 Red-leaved gage	203 ± 10

(2017) reported that their levels in cherry kernels ranged from 1307.65 µg/g to 2882.76 µg/g, 425.32 µg/g to 1372.82 µg/g and 14.92 µg/g to 35.03 µg/g, respectively. Similar concentrations of amygdalin in red and black cherry seeds were reported by Bolarinwa et al. (2014). These values were considerably higher than those obtained in our study when expressed as total cyanide content on a dry weight basis and can be attributed to higher cyanide concentrations in the seeds compared to the whole pits used in our study. Lee et al. (2017) found significantly higher levels of amygdalin in seeds compared to the endocarp and mesocarp of peach fruit.

As can be seen in Table 2, the cyanide content in the pits of the plum accessions also varied noticeably, ranging from 35.6 µg/g (3576 plum) to 253 µg/g (Natural hybrid Vrbanska). Plum accession 3576 is a planned cross, while Natural hybrid Vrbanska is a primitive genotype. The highest mean cyanide content (162 µg/g) was determined in pits of wild genotypes, followed by cultivated accessions (87.3 µg/g) and planned crosses (51.8 µg/g). The cyanide content of all plum pits in our study is lower than the average values (764 mg HCN/g, 377.6 mg HCN/g) reported by Haque and Bradbury (2002) and Sheikh et al. (2021) for plum kernel samples. Bolarinwa (2014) showed that the amygdalin content in plum seeds depends mainly on the variety.

Total cyanide levels in wild genotypes of gages ranged from 124 µg/g (Gage K-9) to 310 µg/g (Gage 6319). We also included two accessions from a planned cross, whose cyanide levels ranged from 58.6 µg/g (A.I. 3 gage) to 203 µg/g (A.I. 8 red-leaved gage) (Table 2). To our knowledge, no studies could be found that included cyanogenic glycoside analysis in any fruit part of gage fruits.

Considerable variation in cyanide content was found within the studied varieties of a fruit tree species, reaching a coefficient of variation of 56.9% for cherries, followed by plums (54.3%) and gages (30.5%) (Table 3). The differences in cyanide content within fruit tree species could be attributed to variety and other factors such as growing practices and environmental conditions, which are generally poorly defined (Vetter, 2000; Ballhorn 2011; Kaack, 2017; Lee et al., 2017).

Differences in cyanide content in pits of three fruit species

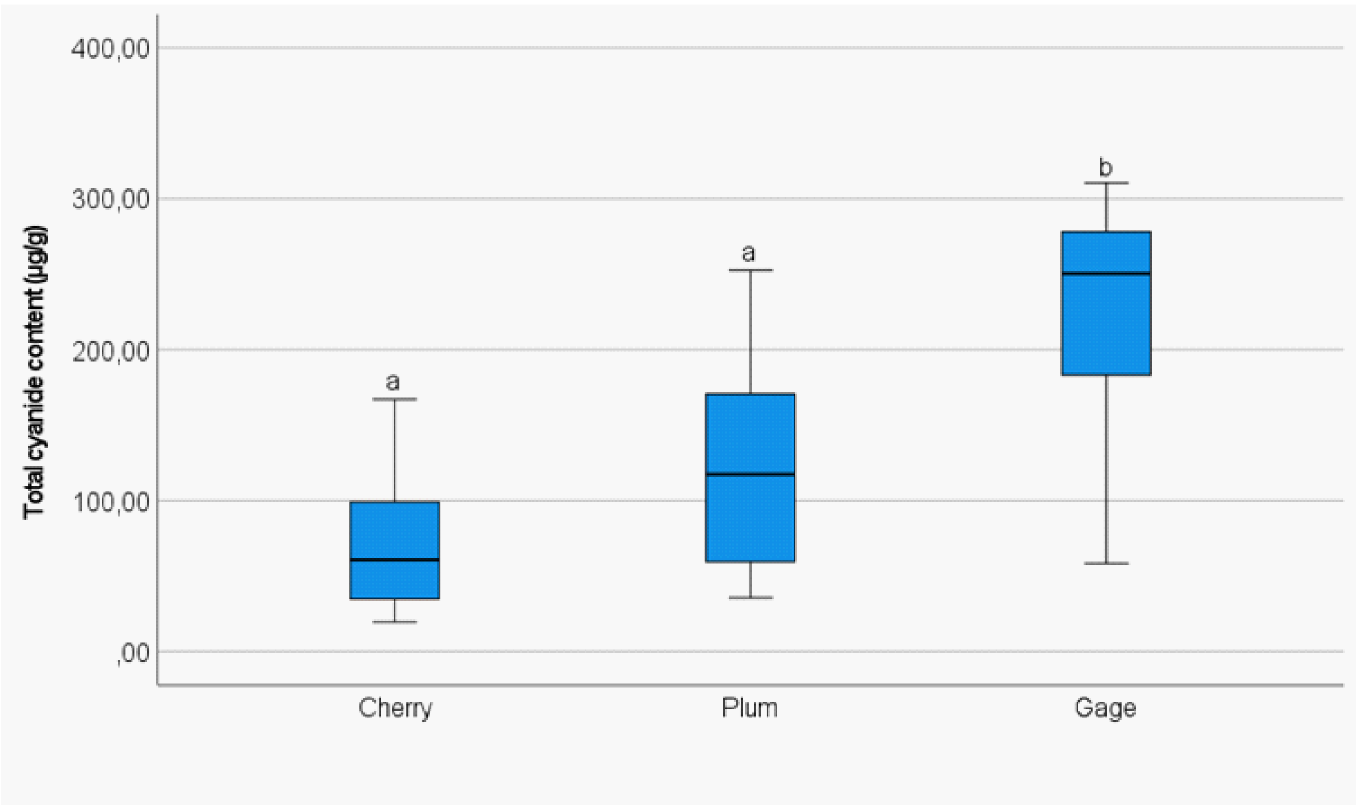
The results of the descriptive statistics for the three fruit species are shown in Table 3. Gage pits contained the most cyanide (*me* = 250 µg/g), followed by plums (*me* = 118 µg/g) and cherries (*me* = 61.0 µg/g). The Kruskal-Wallis test showed statistical significance of fruit species on total cyanide content ($p < 0.001$). We also performed a multiple comparison test, which revealed a statistically significant difference in total cyanide content between the medians of cherries and gages and plums and gages, while no statistically significant differences were found between cherries and plums (Table 3). We used the medians for analysis because the distribution of gage samples was abnormal, as shown in Figure 1 (also tested using Shapiro Wilk test, sig.=0.037).

Among the fruit species included in our study, plums are the most commonly used fruit for the production of spirits and liqueurs. Small amounts of cyanogenic glycosides present in plums and other species contribute to the distinctive flavors and qualities of spirits and liqueurs, but may be harmful to human health at higher concentrations. The content of CGs in spirits depends mainly on the amount present in the raw material (Śliwińska et al., 2015; Senica et al., 2016; Senica et al., 2017). According to our results, the transfer of cyanides

Table 3: Descriptive statistics of cyanide content in gages, plums and cherries accessions (μg/g)

	Gages	Plums	Cherries
n	20	20	30
min	58.6	35.6	19.6
max	310	253	167
$\bar{x}$	228	116	68.4
SD	69.6	14.0	38.9
me	250	118	61.0
CV (%)	30.5	54.3	56.9
Percentile			
25	180	58.9	33.3
50	250	118	61.0
75	279	172	99.4

n - total number of samples, min - minimum value, max - maximum value, $\bar{X}$ - average value, SD - standard deviation, me - median, CV(%) - coefficient of variation



Medians (n=20 for gages and plums, and 30 for cherries) labeled with the same letter are not significantly different (Dunn-Bonferroni post hoc test, $p < 0.05$).

Figure 1: Comparison of medians and sample distributions of three fruit species

from cherry pits should not pose a risk in the production of cherry liqueurs, as their average concentrations are up to 2 times lower compared to plums and 4 times lower compared to gages. The results also suggest that the cultivars 'Hanita', 'Ruth Gerstetter', and accessions R x L 105, R x L 98, R x L 120, gage K-9, purple gage and A.I. 3 gage may be more suitable for whole fruit processing, including pits, because of their low total cyanide content. On the other hand, caution should be considered when making liqueurs or tinctures from fruits of wild cultivars, as they may contain up to 5 times more cyanide than cultivated genotypes such as *P. domestica* 'Hanita' and *P. domestica* 'Ruth Gerstetter', which are commonly available in nurseries throughout Europe.

CONCLUSION

Analysis of cyanide content in the pits of various plum, cherry, and gage accessions revealed considerable variability both within and among fruit species. Furthermore, total cyanide content can vary considerably even within the same crosses. In view of our results, the R x L 98 (cherry), 3576 (plum) and A.I.3 gage accessions may be more suitable for processing because of their low cyanide content. Further research should focus on the determination of cyanide content in plum and cherry pits of the varieties most commonly used for spirit and liqueur production, as well as on the transfer of compounds from the fruit to other end products.

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Uporaba pikratne metode za določitev vsebnosti cianida v koščicah češenj, ringlojev in sliv

IZVLEČEK

Cianogeni glikozidi so prisotni v vsaj 2500 taksonomskih skupinah različnih rastlinskih družin, ki vključujejo številne gospodarsko pomembne sadne vrste. Koncentrirajo se v semenih plodov in lahko predstavljajo tveganje za zdravje ljudi, ker se pri hidrolizi sprošča cianid. V naši raziskavi smo s pikratno metodo določili vsebnost skupnega cianida v koščicah češenj (*Prunus avium* L.), sliv (*Prunus domestica* L.) in ringlojev (*Prunus domestica* subsp. *italica* (Borkh.) Gams). Najnižjo vsebnost (19,6 µg/g) smo izmerili v koščicah akcesije češnje R x L 98 in najvišjo (310 µg/g) v akcesiji ringloja 6319. Znatne razlike v vsebnosti cianida smo zabeležili tudi znotraj sadnih vrst s koeficienti variacije 56,9 % pri češnjah, 54,4 % pri slivah in 30,5 % pri ringlojih. Primerjava srednjih vrednosti znotraj sadnih vrst je pokazala, da je vsebnost cianida v koščicah ringlojev statistično značilno višja kot v koščicah sliv in češenj.

Ključne besede: cianid, koščice, češnje, ringloji, slive