Ethanol Biosynthesis and Hydrocyanic Acid Liberation During Fruit Mashes Fermentation

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Abstract

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The biosynthesis of ethanol and release of hydrocyanic acid are important factors characterising the fermentation process of fruit mashes. The effects were determined of the fruit pretreatment and method of fermentation (with or without the addition of wine yeast) on the dynamics of ethanol biosynthesis and hydrocyanic acid liberation during fruit mashes fermentation and its concentrations in the spirits obtained. Depending on the fermentation variants, the highest rate of ethanol biosynthesis was observed between the first and third days of plum mashes fermentation, and between the first and fourth days of chokeberry mashes fermentation. In the majority of fermented mashes, the maximum dynamics of HCN liberation was recorded on the first day of the process. Spontaneous fermentation of fruit pulp resulted in much higher amounts of HCN in the spirits obtained (10.5 mg/l of plum spirit 40%, v/v, and 28.90 mg/l of chokeberry spirit 40%, v/v) in relation to the contents observed in the distillates from the mashes fermented with the addition of *S. bayanus* wine yeast (2.80 mg/l of plum spirit 40%, v/v, and 12.46 mg/l of chokeberry spirit 40%,v/v). The treatment with the pectolytic preparation (PEKTOZYME™ POWERMash) had no significant effect on the concentration of HCN in fruit spirits, whereas the pressure/thermal treatment reduced HCN content, especially in chokeberry distillate, by ca. 71% as compared to the reference spirit (obtained from raw pulp).

Keywords: cyanogenic glycosides; fruit spirits; plum; black chokeberry; *Aronia melanocarpa*; fermentation

Hydrocyanic acid (HCN), referred to as hydrogen cyanide, is formed as a result of enzymatic hydrolysis of cyanogenic glycosides which are produced by various plant species as secondary metabolites. Cyanogenic glycosides present in plants are relatively nontoxic until HCN is released (EFSA 2004). In intact plants, these compounds are accumulated in the cell vacuoles. Therefore, they are spatially separated from specific β-glucosidases which are located in the apoplast. Crushing the plant materials either by means of technical processes or chewing by animals results in cell disintegration and initiates enzymatic hydrolysis of cyanogenic compounds by β-glucosidases (EC 3.2.1.21), resulting in the formation of sugars and cyanohydrin. Cyanohydrins (α-hydroxynitriles) can decompose spontaneously or in the process of enzymatic reaction catalysed by hydroxynitrile lyase (EC 4.1.2.37) resulting in the formation of a ketone or an aldehyde and HCN (Brimer 2001). According to Ballhorn *et al.* (2010), three cyanogenic features, i.e., cyanogenic potential (HCNp; concentration of cyanogenic precursors), β-glucosidase activity – present both in the plant material and in the "consumers" digestive tract, and cyanogenic capacity (HCNc;

release of cyanide per time unit) affect the potential toxicity of the cyanogenic plants.

For brandies produced from stone fruit spirits, consumers often desire the typical "bitter almond" taste. However, the positive flavour compounds introduced from the stones may be accompanied by detrimental influences and even health risks. The fermentation of fruits containing cyanogenic glycosides and the subsequent spirit production have been claimed to be a frequent cause of the formation of carcinogenic ethyl carbamate (Ough 1976; Ough *et al.* 1988; Aresta *et al.* 2001; Lachenmeier *et al.* 2005; Lachenmeier 2007).

The degradation of the commonly occurring cyanogenic glycosides from *Prunus* sp., prunasin and amygdalin, leads to the liberation of hydrocyanic acid and benzaldehyde. Benzaldehyde is generally regarded as safe as a food additive in the United States and is accepted as a flavouring substance by the European Union (EFSA 2007), whereas the maximum level of HCN in foodstuffs and beverages is strictly limited (Council Directive No. 88/388/EEC 1988).

Regulation (EC) No. 110 (2008) of both the European Parliament and the Council on the definition, description, presentation, labelling, and protection of geographical indications of spirit drinks, repealing Council Regulation (EEC) No. 1576/89, stipulates that the maximum hydrocyanic acid content in stone fruit spirits and stone fruit mark spirits shall amount to 7 g/hl of 100% (v/v) alcohol (70 mg/l).

Very popular among Eastern and Central European stone fruit brandies are plum brandies (slivovitz) and in a submontane region of Poland with a specific climate, Śliwowica Łącka (Satora & Tuszyński 2008).

Our previous studies (Balcerek & Szopa 2002; 2005) demonstrated that the black chokeberry (*Aronia melanocarpa* Elliot) represents a very interesting raw material for the production of natural spirit. Both plum fruit and black chokeberry contain cyanogenic glycosides, and their hydrolysis during alcoholic fermentation results in the liberation of hydrocyanic acid.

The aim of the present work was to determine the effect of the fruit pretreatment and method of fermentation (with or without the addition of wine yeast) on the dynamics of ethanol biosynthesis and hydrocyanic acid liberation during fruit mashes fermentation, and to quantify the concentration of the latter in the spirits obtained.

MATERIAL AND METHODS

Raw material and mashing process. The raw materials for the production of fruit spirits were mashes prepared from plums cv. Węgierka Zwykła and black chokeberry (*Aronia melanocarpa* Elliot). To prepare the mashes for fermentation, the fruits (stoned plums and whole black chokeberry) were homogenised into pulp. Non-comminuted stones in the amount of 10% by wt were added to the plum pulp. All mashes were supplemented with $(NH_4)_2HPO_4$ (0.2 g/kg fruit pulp). The variants of the fruit pretreatment and method of fermentation (with or without addition of yeast) were as follows:

A – raw fruit pulp, *S. bayanus* wine yeast (0.3 g DM/kg mash),

B – raw fruit pulp, spontaneous fermentation

C – fruit pulp subjected to pectolytic action (PEKTOZYME™ POWERMash; Danisco A/S, Grindsted, Denmark – 0.07 ml/kg pulp, at 20 ÷ 22°C), *S. bayanus* wine yeast (0.3 g DM/kg mash),

D – fruit pulp after pressure/thermal treatment (121°C, 0.1 MPa, 20 min), *S. bayanus* wine yeast (0.3 g DM/kg mash).

Fermentation of the mashes (ca. 8 kg) was carried out in flat-bottomed flasks of a volume of 10 litres. The flasks were closed with stoppers, equipped with fermentation pipes containing glycerol. Fermentation was conducted at 28 ÷ 30°C. The samples of fermented mashes were collected every day to determine the contents of ethanol and hydrocyanic acid. The process was continued until the content of ethanol remained stable.

Distillation of total alcohol from mashes after fermentation was carried out by using a distillation unit including a boiling flask, a Liebig condenser. and a receiving flask. The obtained raw spirits containing 18.5–24.5% v/v ethanol were then refined up to approximately 42% (v/v) ethanol in a distillation apparatus equipped with a bi-rectifier unit (dephlegmator according to Golodetz) (Young 1922) and subjected to chemical analyses.

Determination of cyanogenic glycosides in fresh fruit. A sample of 10÷20 g fruit pulp or 1÷2 g seeds removed from stones of fresh plum was ground and immediately transferred into a distillation flask, and 5 ml of 50% (v/v) *o*-phosphoric acid solution and ca. 250 ml of distilled water were added. HCN liberated was distilled at 100°C for 1 h in a vacuum distillation apparatus (according

to HACH Company (Loveland, USA) and trapped in a receiver with 50 ml of 0.1 mol/l NaOH solution. The distillates obtained were transferred quantitatively into a calibrated flask and made up with to the volume of 100 ml. HCN concentration was determined as described below. Assuming the cyanogenic glycoside of mature plum seeds (Voldřich & Kyzlink 1992) and of black chokeberry fruits (Lehman 1990) was principally amygdalin, the liberated HCN was expressed as amygdalin content, using a calibration curve prepared with amygdalin aqueous standard solutions ranging from 0.5 mg to 10.0 mg HCN equivalents/l. Similarly, prunasin content in the pulp of fresh plums (Voldřich & Kyzlink 1992) was calculated from HCN content of the pulp, using a calibration curve prepared from prunasin aqueous standard solutions containing from 0.005 mg to 0.5 mg HCN equivalents/litre.

HCN content determination. Free HCN content was determined spectrophotometrically using pyridine-pyrazolon reagents. The method involves the conversion of HCN to cyanogen chloride with chloramine T solution. As a result of the reaction of this compound with a mixture of pyridine containing 1-phenyl-3-methyl-5-pyrazolone and 4.4'-bis(1-phenyl-3-methyl-5-pyrazolone), a coloured complex is formed, which was spectrophotometrically measured at a wavelength of 490 nm. The amount of cyanide in the samples was quantified by using standard solutions prepared from NaCN, ranging from 0 mg to 1 mg HCN equivalents/l (Epstein 1947; Hach Company 2000).

Total hydrocyanic acid (the sum of free and bound HCN) in the spirits obtained was determined after acid hydrolysis with 50% *o*-phosphoric acid, by means of the method described above. HCN liberated during fruit mash fermentation was separated from the matrix by means of distillation without acid hydrolysis. The content of HCN was determined by using cyanide test kit purchased from Hach Company (Loveland, USA). All other reagents used were of analytical-reagent grade.

Statistical analysis. The determination of standard deviations and Student's *t* test at the significance level of $\alpha = 0.05$ were carried out by using Origin 6.0 software. All assays were carried out in triplicate.

Calculations. The rate of ethanol biosynthesis in the tested fruit mashes ($V_{C_2H_5OH}$) was expressed in (g/kg/h) and calculated according to the following equation:

$$V_{C_2H_5OH} = (\Delta C_{C_2H_5OH} \times \rho)/t$$

where:

$\Delta C_{C_2H_5OH}$ – increase of absolute ethanol amount produced in successive days of fermentation (ml ethanol 100%, v/v/ kg mash)

ρ – density of absolute ethanol (100% v/v) (0.789 g/ml)

t – fermentation day (24 h)

The rate of HCN liberation in the tested fruit mashes (V_{HCN}) was expressed in (mg/kg/h) and calculated according to the following equation:

$$V_{HCN} = \Delta C_{HCN}/t$$

where:

ΔC_{HCN} – increase of HCN amount liberated in successive days of fermentation (mg HCN/kg mash)

t – fermentation day (24 h)

RESULTS AND DISCUSSION

Cyanogenic glycoside contents were determined in the stones and pulp of plums cv. Węgierka Zwykła used in Poland for slivovitz production and in whole black chokeberry which is a noteworthy raw material for the fruit distillates production (Table 1). The content of prunasin in plum pulp was at the level of 0.25 ± 0.02 g/100 g whereas the amount of amygdalin in stone seeds reached 106.8 ± 5.3 mg/100 g. Black chokeberry fruits contained amygdalin at the level of 70.2 ± 3.5 mg/100 g fruit (Table 1).

The data published by Voldřich and Kyzlink (1992) showed that the seeds of fresh plums (un-

Table 1. Cyanogenic glycoside contents in tested fruits

Compound	Part of fruits	Plum cv. Węgierka Zwykła	Black chokeberry
Cyanogenic glycoside (mg/100 g)	pulp (prunasin)	0.25 ± 0.02	70.2 ± 3.5*
	seeds of stones (amygdalin)	106.8 ± 5.3	

*whole fruits (content of glycoside expressed as amygdalin)

known cultivar) contained 0.26% by wt of amygdalin whereas prunasin content in plum pulp was at the level of 9.80 mg/kg. Compared to the results of our study, Lehman (1990) found much lower contents of amygdalin in black chokeberry (20.13 mg/100 g of whole fruit, 5.75 mg/100 g of juice, 52.30–87.70 mg/100 g of pressing-cake). The differences observed between the content of cyanogenic glycosides in fruits used in our study and the data presented in the literature may be attributed to various factors such as differences between cultivars (Voldřich & Kyzlink 1992; Vetter 2000), climatic conditions, agricultural technology as well as fruit ripeness (Knight & Walter 2001; Lewis & Elvin-Lewis 2003).

The rate of ethanol biosynthesis and hydrocyanic acid liberation during fruit mashes fermentation

Maximum ethanol concentrations (5.0 ± 0.3%, v/v, in plum mash, 3.4 ± 0.2%, v/v, in chokeberry mash) during fermentation of raw fruit mashes by *Saccharomyces bayanus* wine yeast were observed on the eighth fermentation day (Figures 1a and 2a). However, the highest rate of ethanol biosynthesis was observed on the third day of the process and ranged from 0.33 ± 0.02 g/kg/h in the chokeberry mash to 0.69 ± 0.03 g/kg/h in the plum mash (Figures 1b and 2b). The dynamics of ethanol synthesis consecutively decreased during the successive days of fermentation despite the increase of its concentration in the fermented mashes.

A longer initial phase was observed during spontaneous fermentation of raw fruit pulp. The synthesis of ethanol started on the second day of the process and the highest rates (0.49 ± 0.02 g/kg/h in plum mash, 0.36 ± 0.02 g/kg/h in chokeberry mash) were observed on the third and fourth days, respectively (Figures 1b and 2b). Over the following days, a subsequent increase of ethanol concentration was noted (Figures 1a and 3a) but the dynamics of its production was much slower. The completion of plum mash fermentation was observed after thirteen days (ethanol concentration 4.3 ± 0.2%, v/v) (Figure 1a) whereas the fermentation of chokeberry mash stopped after fifteen days (ethanol concentration 3.3 ± 0.02%, v/v) (Figure 2a).

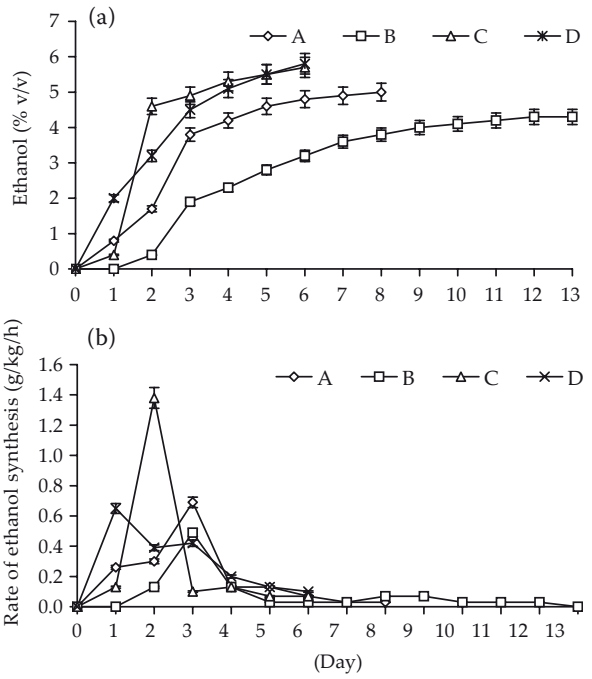


Figure 1. (a) The changes of ethanol contents and (b) the rate of ethanol biosynthesis during plum mashes fermentation

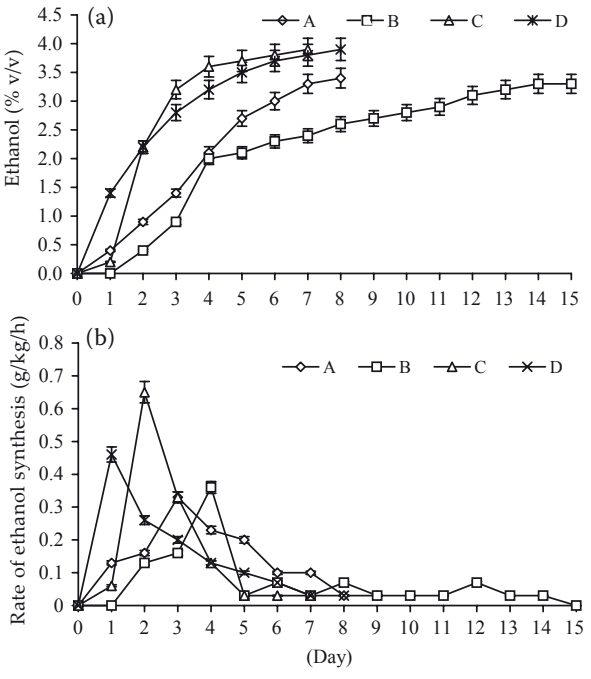


Figure 2. (a) The changes of ethanol contents and (b) the rate of ethanol biosynthesis during chokeberry mashes fermentation

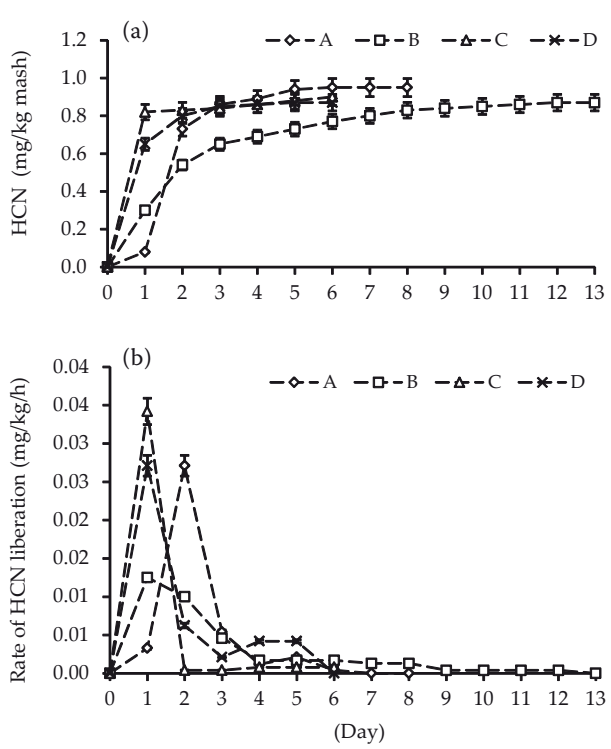
A – raw pulp, *S. bayanus*; B – raw pulp, spontaneous fermentation; C – pulp after treatment with Pectozyme™ POWERMash, *S. bayanus*; D – pulp after pressure/thermal treatment, *S. bayanus*

Depectinisation of plum and chokeberry pulp due to hydrolysis of pectins resulted in slackening and liquefaction of fruit mashes. The pectolytic action resulted in higher dynamics of fruit fermentation in comparison with that of the raw fruit pulp. The highest production of ethanol was observed on the second day of fermentation and reached the level of 1.38 ± 0.07 mg/kg/h in plum mash and 0.65 ± 0.03 mg/kg/h in chokeberry mash, respectively (Figures 1b and 2b).

Fruit pulp fermentation after pressure/thermal treatment showed maximum rate of ethanol synthesis during the first day of the process (0.65 ± 0.03 g/kg/h in plum pulp and 0.46 ± 0.02 g/kg/h in chokeberry pulp) (Figures 1b and 2b). On the following days, the dynamics of ethanol production was much slower despite the subsequent increase of its concentration. The completion of plum mash fermentation was observed after six days (ethanol concentration 5.8 ± 0.3%, v/v) whereas the fermentation of chokeberry mash stopped after eight days (ethanol concentration 3.9 ± 0.2%, v/v) (Figures 1a and 2a).

Quantitative analysis of hydrocyanic acid liberated during fruit mashes fermentation showed that HCN amounts formed in chokeberry mashes were higher then those formed in the plum mashes (Figures 3a and 4a). HCN content in black chokeberry mash after fermentation with *S. bayanus* yeast reached 4.80 ± 0.2 mg/kg (Figure 4a) whereas in the plum mash it was about 5-fold lower (0.95 ± 0.05 mg/kg) (Figure 3a). The differences between the amounts of liberated HCN may have been caused by the addition of non-comminuted stones to the plum mashes. The liberation of this compound might have been influenced also by a diversified concentration of the cyanogenic precursors, β-glucosidase activity in the plant material, and the rate of cyanide release (Stochmal & Oleszek 1997; Niedźwiedź-Siegień 1998; Niedźwiedź-Siegień & Gierasimiuk 2001; Ballhorn *et al.* 2005).

The highest rate of HCN release during the fermentation of raw plum pulp with *S. bayanus* yeast was observed at the initial period of fermentation, especially on the second day (0.027 ± 0.001 mg/kg/h)



A – raw pulp, *S. bayanus*; B – raw pulp, spontaneous fermentation; C – pulp after treatment with Pectozyme™ POWERMash, *S. bayanus*; D – pulp after pressure/thermal treatment, *S. bayanus*

Figure 3. (a) The changes of HCN contents during plum and (b) the rate of HCN liberation during chokeberry-mashes fermentation

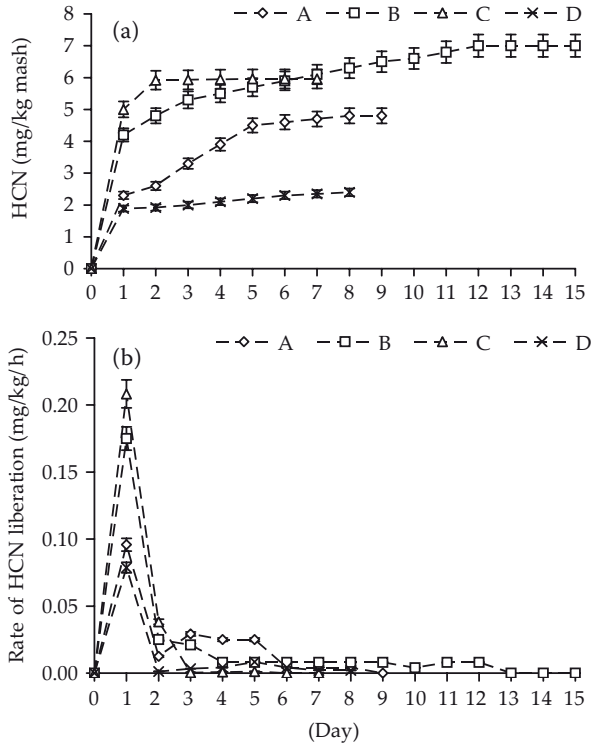


Figure 4. (a) The changes of HCN contents during chokeberry mashes fermentation and (b) the rate of HCN liberation

(Figure 3b), and on the first day in the case of raw chokeberry pulp fermentation (0.096 ± 0.005 mg/kg/h) (Figure 4b). A significant decrease of the rate of cyanogenic glycosides hydrolysis and HCN liberation (especially in the plum pulp) during the following periods of the fruit mashes fermentation was observed despite the increase of its total concentration in the fermented mashes.

During spontaneous fermentation of fruit mashes, maximum dynamics of HCN liberation was observed on the first day, before pre-fermentation of fruits. The rate of HCN liberation in the fermented plum mash reached 0.013±0.001 mg/kg/h (Figure 3b) while in chokeberry mash it was more than 10-fold higher (0.175 ± 0.009 mg/kg/h) (Figure 4b). These findings are presumably due to the higher cyanogenic capacity in black chokeberry fruits.

There were no statistically significant differences ($P > 0.2$) in the concentration of hydrocyanic acid between plum mash fermented with the addition of wine yeast and spontaneously fermented pulp (Figure 3a). In contrast to plum mash, the content of HCN in black chokeberry mash after fermentation with indigenous yeast reached 7.0 ± 0.4 mg/kg and was by ca. 46% higher as compared to that observed in mash fermented by *S. bayanus* wine yeast (Figure 4a).

Maximum dynamics of HCN liberation was noted on the first day of fermentation of both mashes treated with a pectolytic preparation and spontaneously fermented mashes prepared from raw fruits. However, the catalytic action of the preparation employed contributed presumably not only to a rapid hydrolysis of pectins but also caused the liberation of significant amounts of HCN during the first day of the process. HCN level varied from ca. 84% (black chokeberry mash) to 91% (plum mash) of its total amount determined after the completion of the process. An interesting phenomenon is that the total amount of HCN liberated during the fermentation of the plum pulp treated with PEKTOZYME™ POWERMash was not statistically different ($P > 0.2$) compared to the mash without pectolytic treatment (raw fruit pulp) (Figure 3a). The content of hydrocyanic acid in chokeberry mash digested with PEKTOZYME™ POWERMash preparation was ca. 24% higher ($0.001 < P < 0.01$) than in the reference mash (Figure 4a). Our results are consistent with the findings reported by Pogorzelski (1982, 1990), who also observed a catalytic effect of the pectolytic preparation action on the rate of cyanogenic glycosides hydrolysis.

Our present study revealed that the treatment of plum pulp with PEKTOZYME™ POWERMash preparation did not result in a higher concentration of HCN in comparison with the reference mash (Figure 3a) whereas the digestion of fruit pulp with Pektopol PT-400 preparation carried out in our previous works (Balcerek *et al.* 2003; Balcerek & Szopa 2006) resulted in a significant increase of HCN content in plum and blackthorn mashes and spirits.

One of the reasons for the differences between the amounts of HCN liberated during fermentation of fruit mashes digested with various pectolytic preparations could be a different combination of enzymes present in the preparations used. For example, PEKTOPOL PT-400 (Pektowin Jasło Inc., Jasło, Poland) is a preparation containing a pectolytic enzyme complex, obtained through a of biosynthetic process with the use of *Aspergillus niger*. The pectolytic enzymes in the complex (polygalacturonases, pectinesterases, pectate liases) decompose pectin in pulps and juices. The preparation contains also cellulases, hemicellulases, proteases, and other enzymes which hydrolyse polysaccharides and proteins, thus supporting the processing of pulps. PEKTOZYME POWERMash (Danisco A/S, Grindsted, Denmark) is a product for fruit mash enzymation at ambient temperature. It is also produced by *Aspergillus niger* and delivers outstanding results with improved juice yield and press capacity. Moreover, the parameters of the treatment with the preparations studied could have an effect on the level of liberated hydrocyanic acid. The treatment with Pektopol PT-400 preparation was carried out at the temperature of 50 ÷ 55°C (Balcerek *et al.* 2003; Balcerek & Szopa 2006), while the digestion of the tested fruit mashes with PEKTOZYME™ POWERMash preparation was performed at the temperature of 20 ÷ 22°C.

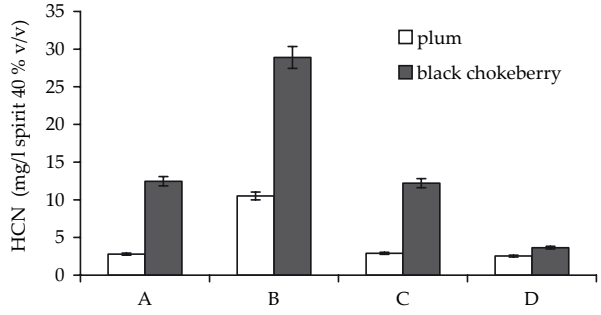
Maximum rate of HCN liberation during fermentation of fruit pulp submitted to the pressure/thermal treatment was observed during a pre-fermentation phase and reached the level of 0.027 ± 0.001 mg/kg/h in plum mash whereas in chokeberry mash it was significantly higher (0.079 ± 0.004 mg/kg/h) (Figures 3b and 4b). The content of HCN in mashes, after the completion of fermentation, ranged between 0.87 ± 0.04 (plum) and 2.40 ± 0.12 mg/kg (chokeberry) (Figures 3a and 4a). Our study indicated that the application of the pressure/thermal treatment caused a sta-

tistically significant ($P < 0.001$) decrease of HCN liberated in chokeberry mash, which was by 50% lower as compared to that observed in the mash prepared from raw fruit pulp (Figure 4a). The results obtained are in accordance with the study of Pogorzelski (1982; 1990). An increased temperature (121°C) causes the inactivation of native enzymes which catalyse hydrolysis of cyanogenic glycosides present in the fruit pulp. Also Voldřich and Kyzlink (1992) reported on the beneficial effects of HTST method used for the treatment of fruits with a higher cyanogenic glycoside content on the decrease of HCN amount.

Due to the use of non-comminuted stones in the plum mashes preparation, their pressure/thermal treatment did not change the content of HCN liberated after the fermentation was completed, in comparison with the control mash (prepared from raw pulp) ($P > 0.2$) (Figure 3a).

The hydrocyanic acid content in the spirits obtained

The tested plum and black chokeberry spirits contained significantly different amounts of free hydrocyanic acid, ranging from 2.54 ± 0.13 mg/l to 10.50 ± 0.53 mg/l in plum spirit 40% (v/v), and from 3.65 ± 0.18 mg/l to 28.90 ± 1.44 mg/l in chokeberry spirits 40% (v/v) (Figure 5). Our study shows that none of the tested samples of fruit spirits contained HCN in bound form and the data are in line with the findings of Procházka *et al.* (1988) who also reported the presence of only free HCN in the tested fruit distillates.



A – raw pulp, *S. bayanus*; B – raw pulp, spontaneous fermentation; C – pulp after treatment with Pectozyme™ POWERMash, *S. bayanus*; D – pulp after pressure/thermal treatment, *S. bayanus*

Figure 5. The hydrocyanic acid contents in the fruit spirits obtained

The concentrations of HCN in plum spirits derived from the mashes prepared from raw pulp as well as from fruits treated with PEKTOZYME™ POWERMash were similar ($P > 0.20$) and ranged between 2.80 ± 0.14 mg/l and 2.90 ± 0.15 mg/l spirit 40% (v/v). The distillate obtained after spontaneous fermentation of raw plum pulp contained a much larger amount of HCN (10.5 ± 0.52 mg/l spirit 40%, v/v).

The concentration of HCN in chokeberry spirits was much higher than that observed in plum distillates. However, similarly to plum spirits, there were no statistically significant differences ($P > 0.20$) between the concentrations of HCN in the distillates obtained both from raw chokeberry pulp and from the mash prepared by using the pectolytic preparation.

Spontaneous fermentation of chokeberry pulp resulted in a significant increase of liberated HCN to 28.90 ± 1.44 mg/l spirit 40% (v/v). The application of the pressure/thermal treatment of black chokeberry pulp caused a substantial decrease in HCN content in the spirit by ca. 71% in relation to the spirit obtained from reference mash (prepared from raw pulp) (Figure 5). The results obtained are consistent with the data published in the literature (Pogorzelski 1982; 1990) and our previous studies (Balcerek *et al.* 2003; Balcerek & Szopa 2006).

The concentration of HCN in plum spirit obtained from the pulp after the pressure/thermal treatment did not result in a statistically lower ($P > 0.20$) amount of HCN as compared to the distillate obtained from the reference mash. We suggest that this phenomenon is due to using non-comminuted stones to prepare the plum mash. According to the data shown in Table 1, the seeds present in the stones are the major source of cyanogenic glycosides.

To conclude, the study on the effect of fruit pretreatment and method of fermentation (with or without yeast addition) demonstrated their different impacts on the dynamics of ethanol biosynthesis and hydrocyanic acid liberation and their contents in the spirits obtained. The selection of a suitable method of fruit mashes preparation and wine yeast addition can lead to obtaining spirits with a good flavour and limited contents of hydrocyanic acid (HCN).

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