



THE EFFECT OF DIFFERENT PRETREATMENTS ON BETAINES SEPARATION

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ABSTRACT: Sugar beet molasses represents the most important by-product in the sugar beet industry which can be valorised through various methods due to very valuable composition. Standard beet molasses composition consists of sugar, nonsugar components and water. Furthermore, beet molasses normally contains betaine in the range from 3 to 8%/DM. Betaine is an important nonsugar component which is not eliminated during the production of sugar, so it accumulates in the molasses and could be obtained through separation process on an industrial chromatographic columns.

The main objective of this research was to increase betaine content in betaine fractions and lower other impurities by applying different pretreatments before final separation process. Samples composition was characterized by using the HPLC technique and the quantification was performed by applying external standard method with betaine as a standard. Separation techniques used in this research were: (I) separation method without pretreatment (II) separation with preceded acidification (samples pH value adjustment) and (III) separation with alcohol fermentation as previous treatment. Betaine content in the starting sample was 62.76%/DM. The highest increase in betaine content was achieved by using separation method without pretreatment having final betaine content of 85.16%/DM. By using acidification as pretreatment before centrifugation the level of betaine was increased up to 67.68%/DM, whereas implementing the alcohol fermentation before centrifugation showed no significant differences compared to the starting sample.

Key words: *sugar beet molasses, betaine fraction, acidification, alcohol fermentation, separation techniques, HPLC*

INTRODUCTION

Molasses represents the most important by-product of sugar beet and sugar cane processing industry, due to valuable composition and a numerous possibilities for further use (Asadi, 2007). The molasses is consisting of sucrose (50%), nonsugars (30%) and water (20%) (Šušić et al., 1995). Composition and quality of molasses is not the same every year, it depends mostly on quality of sugar beet as a raw material, applied processing technology

(juice purification stage, crystallization process), area of sugar beet cultivation and used fertilizer (Ramm-Schmit, 1998; Higginbotham and McCarthy, 1998; Šušić et al., 1995). The most abundant nitrogenous compound (nonsugar) present in sugar beet molasses, but absent in sugar cane molasses, is betaine (Craig, 2004). Betaine has a lot of different sources such as cereals, spinach, quinoa, but sugar beet molasses represents the most plen-

tiful source of betaine with around 3-8 %/DM. Betaine is not destroyed nor eliminated during processing of crystalline sugar from sugar beet and it accumulates in molasses almost without loss (Krulj et al., 2014; Bennett, 1942). Generally, level of betaine in sugar beet peaks during the autumn months whereas growth under drought yields betaine levels in sugar beet (Groom et al., 2009; Corol et al., 2012).

Betaine (betaine glycine, trimethylglycine) is a quaternary amine and has zwitterionic structure. As a high polar compound, betaine is soluble in water, same as in methanol (Rivoira et al., 2017). Earlier research has shown that betaine has numerous benefits to the human organism (Craig, 2004). Due to specific chemical structure with three methyl groups, betaine, as a methyl donor, contributes to the remethylation of homocysteine to methionine and the normal function of the homocysteine cycle (Craig, 2004).

Betaine as an osmolyte provides less osmotic stress to cells and protects them from high oscillations in osmotic pressure (Polat and Beklevik, 2001). Betaine glycine has claimed confirmation as GRAS (Generally Recognized as Safe) ingredient. According to positive effects on human organism while eating food enriched with betaine that contains at least 500 mg betaine per portion, betaine has been adopted for use in foods by European Commission (Commission Regulation EU 432. 2012) (Filipčev et al., 2015). Furthermore, Filipčev et al. (2015) suggests that it is advisable to include beet molasses in bread and biscuit products in order to improve the diet of those who follow the gluten-free or vegan diet.

Nowadays, due to growing demand for betaine, sugar industries developed an additional process for molasses separation to several fractions, including betaine fraction. The invention relates to new useful improvement in the recovery of betaine and gives high yield of substantially pure betaine. This could be successfully achieved on industrial chromatographic column and process is called molasses desugaring process (Asadi, 2007). An industrial chromatographic column is filled with ion-

exchange resin. Corresponding ion-exchange process represents a simple principle of ions rejection (nonsugars) and absorption of nonionic compounds (sugar) (Ramm-Schmidt, 1988). Hence, three fractions are separated: raffinate-nonsugar rich fraction, extract-sugar rich fraction and betaine fraction-fraction rich in betaine (Asadi, 2007). Level of betaine in betaine rich fraction can reach up to 90%/DM using chromatographic separation which was conducted in two steps: first "crude" separation and the second "fine" separation.

Other applied techniques were the evaporation under vacuum and crystallization of anhydrous betaine in several steps. Firstly, betaine fractions evaporated to the 80%/DM then crystallized with anhydrous betaine crystals. Afterwards, centrifugation was used in order to separate mother liquor from the crystals. Finally, second crystallization performed providing pure betaine product (Bennett, 1942). In Serbia, only one sugar company has implemented additional processing step with only one ion-exchange column and betaine level obtained by the corresponding procedure reaches up to 70%/DM.

The main objective of this study was to enhance betaine separation by applying different pretreatment techniques in order to recover as much as possible betaine from the fraction.

MATERIALS AND METHODS

Starting sample for all of the experiments was betaine fraction obtained from sugar company Sunoko-Pećinci, Serbia. Acids used for pH variation were 50% citric acid (Betahem, Belgrade, Serbia) and concentrated sulfuric acid (Lachner, Neratovice, Czech Republic). Dry yeast (*Saccharomyces cerevisiae*) was used for biochemical reaction (safeale US-05, Fermentis, France). In the conducted HPLC analysis methanol (UHPLC quality, PanReac AppliChem, Barcelona, Spain), acetonitrile (UHPLC quality, PanReac AppliChem, Barcelona, Spain) and 10mM ammonium acetate buffer pH=3.7 (prepared with ammonium acetate, 99% purity (Lach-Ner, Neratovice, Czech Republic)

and ultrapure water produced with Simplicity UV system were used (Millipore Bedford, MA, USA) as a mobile phases. Betaine anhydrous standard (98% purity, AlfaAesar GmbH&KG, Karlsruhe, Germany) was used for calibration curve construction.

Separation method without pretreatment

The starting sample was transferred into the centrifugation tubes in order to be separated by a centrifugal force (Tehnica LC-320) at a speed of 3500 rpm for 20 minutes at a room temperature (Restu et al., 2015). The supernatant was separated from sediment and was used for the purpose of further analysis.

Separation with preceded acidification

Different pH values were used in order to change the ionic composition of the sample and to force some ionized components to be separated from the others. Starting pH value of betaine fraction sample was 10.6. Adjusted pH values used in the further analysis were: 5.0, 3.7, 3.3, 3.0, 1.5 and 0.7. The adjusted values had significant influence on the betaine ionic composition as previously reported by Kojić et al.(2017), Escudero and Ruiz (2011), and Kim et al.(2009). Precipitate and floculates formed afterwards were centrifuged in order to separate the liquid sample.

Separation with alcohol fermentation as pretreatment

Anaerobic alcohol fermentation was performed by using dry yeast (*Saccharomyces cerevisiae*) in order to ferment present monosaccharides and disaccharides into alcohol and carbon dioxide (Auerman, 1979; Nikolić, 2009). Fermentation was conducted in a laboratory reactor with an extension which provides anaerobic conditions and at a room temperature for 7 days (Jokić et al., 2012). Amount of added yeast was 0.12 g into 100 g of betaine fraction. Afterwards the sample was decanted from the precipitated yeast and forwarded to separation procedure.

HPLC analysis

Before starting the HPLC analysis dry matter content of all samples was deter-

mined for further calculations. Used method for dry substance determination was in accordance with the regulations guided by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA, 2003). Optimized and validated method from Kojić et al. (2017) for estimation of betaine level in cereals and pseudocereals was adopted and applied for the estimation of betaine level in betaine rich fractions obtained from molasses.

A properly homogenized sample (2 g) was weighed and suspended in methanol (25 ml) and after that homogenized on vortex for 10 min. After 30 min of ultrasonication in the ultrasound bath (ATU Ultrasonidos, Valencia, Spain), samples are intensely shaken and placed into the centrifuge for centrifugation for 10 min at the speed of 5000 rpm (Eppendor, Viena, Austria). The upper layer of supernatant (400 µl) was evaporated to dryness. After drying the samples, they were reconstructed in 100 ml ultrapure water and filtrated through a membrane filter (regenerated cellulose, pore size 0.22 µm, diameter 25 mm, Agilent Technologies, Santa Clara, USA).

HPLC system equipped with a Kinetex HILIC[®] column and ELSD detector was used for the betaine level determination. The set flow rate was 0.5 ml/min with mobile phase: acetonitrile and 10 mM acetate buffer at pH 3.7 (80:20 v/v) following isocratic regime. Sample run time was 10 min. Injection volume was 5 µl using auto sampler injection mode and the injection was performed at room temperature. Detection parameters for analysis were: evaporator temperature: 40°C, nebulizer temperature: 55°C, gas flow rate: 1.60 standard litres per minute (SLM) (Kojić et al., 2017).

Afterwards, external calibration method was applied for the quantification of betaine levels in samples. The calibration curve was made from the chromatograms obtained by the addition of anhydrous betaine standard within the range expected in the samples. The betaine area and concentrations were considered as the variables to obtain the linear regression equation. For the betaine quantification the range between 0.05-0.2

mg/mL was used and applied equation was $Y=27762 \cdot X-936.5$ ($R^2=0.9847$, $n=5$), where Y is the peak area and X is the concentration of betaine (mg/ml). All analyses were performed in triplicate. The results were given as mean \pm standard deviation and they were calculated on an amount of dry matter (DM) of each sample.

RESULTS AND DISCUSSION

Results obtained after the HPLC analysis of the betaine samples, indicated that different pretreatments before centrifugation of samples have influence on betaine separation success. The results obtained after the HPLC analysis of the betaine levels in betaine-rich fractions are shown in Table 1.

Figure 1. shows levels of betaine based on dry matter for every sample and the corresponding results are compared with the starting sample.

A significant increase in betaine content with very high betaine purity and little percentage of other components was obtained with samples which were only separated excluding pretreatment (from 62.76% DM to 85.16% DM).

Same conclusion could be taken if recovery yield was observed. Recovery increased from 53.18% to 68.94%. On the other hand smaller increase in betaine levels was detected in the sample with pH 0.7 (from 62.76%/DM to 67.68%/DM), while at other pH values the decrease in betaine levels was detected. No significant change is detected in the samples pretreated by alcohol fermentation before centrifugation (from 62.76%/DM to 63.14%/DM).

An increase in betaine content in samples using centrifugation as pretreatment, up to 35%, occurred due to separation all of the non-betaine particles having higher density and higher molecular masses and thus making liquid phase more concentrated with betaine, which remained dissolved (Rickwood, 2001).

According to the findings of Rivoira et al. (2017), betaine could be present in both liquid (supernatant) and solid matrix,

therefore, complete separation is not possible. However, further analysis will be directed towards identification of the separated precipitate composition.

Applied acidification method as a pretreatment technique provided one positive result. The sample at pH 0.7 had higher betaine content. In the extremely acidic conditions, sucrose hydrolyses while residual peptides denature and form flocs resulting in the higher purity of betaine samples.

Unsatisfactory results with a decrease in betaine level content were acquired with samples at pH 3.0-3.3, which is the isoelectric point of betaine as Kim et. al. (2009) previously showed.

At isoelectric point betaine segregated from a substrate (solvent) (Đaković, 2006). Kojić et al. (2017) reported pH 3.7 as most prominent pH value regarding betaine stability. However, the results of the HPLC analysis showed the reduction in betaine level in the sample at the corresponding pH value.

Samples treated by alcohol fermentation showed discrepancies from the expected values, with no significant changes in betaine level. On the other hand, recovery yield was the lowest with the corresponding technique which could be attributed to very low dry matter of this sample.

No change in betaine level could be explained as a consequence of unfavourable conditions for yeast fermentation: (I) inappropriate pH value (yeast optimal pH value is 4-6); (II) dry substance content was significantly high which limits yeast motion; (III) betaine liquid fraction could have limited source of food (monosaccharides and disaccharides), despite present source of nitrogen, for yeast to reach the fermentation maximum (Nikolić, 2009).

Figure 2 shows betaine peaks in the starting sample and sample prepared by centrifugation method indicating the best result with the highest increase in betaine level content. Retention time is identical for all samples and the position of signals are confirmed by using analytical standard of betaine.

Table 1.

Betaine content in the starting sample and samples with different pretreatment techniques

Sample name	Betaine content (mg/g)	Dry matter (°Bx)	Betaine content (% DM)	Recovery yield (%)
Starting sample	357.73	57	62.76	53.18
Without pretreatment	455.58	57	85.16	68.94
pH= 5	326.28	48.5	60.42	51.73
pH= 3.7	327.08	53.5	57.38	49.84
pH= 3.3	202.91	54	41.48	49.69
pH = 3	196.72	46	42.54	48.70
pH= 1.5	186.37	42	47.71	47.83
pH= 0.7	291.02	43	67.68	47.01
Alcohol fermentation pretreatment	243.09	38.5	63.14	35.69

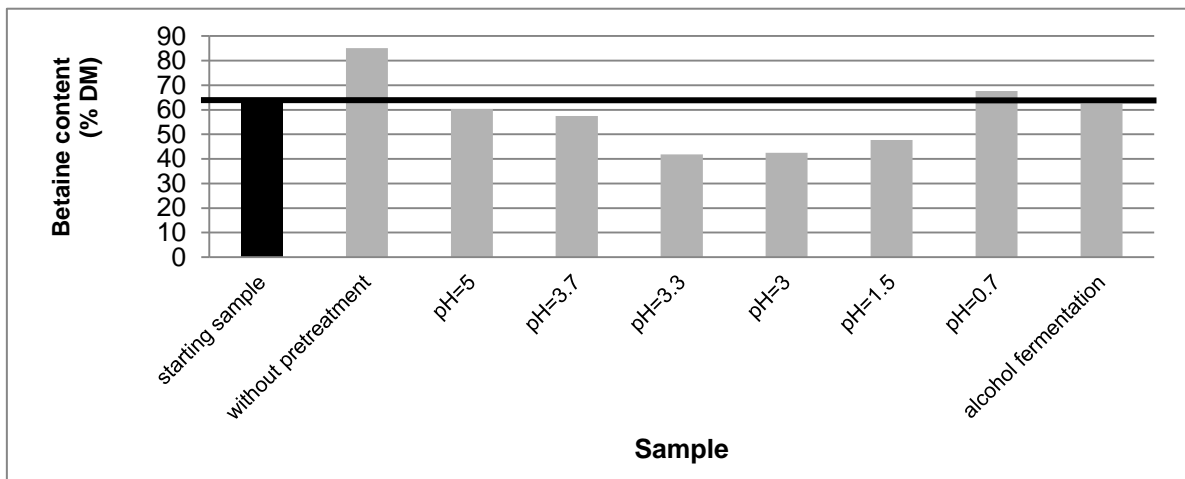


Figure 1. Comparison of betaine levels in all betaine samples with the starting sample

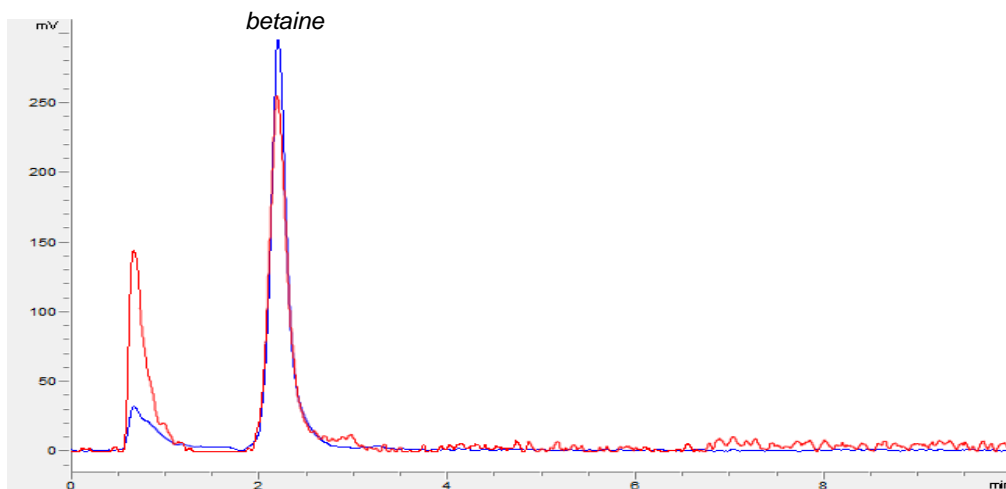


Figure 2. HPLC chromatogram with betaine peak at 2.3 min in the starting sample (red line) and the centrifuged sample without any pretreatment (blue line)

CONCLUSIONS

According to our results, the highest betaine content increase was obtained with the centrifugation technique without any applied pretreatment where interfering particles were separated and levels of betaine in betaine rich fraction increased from 62.76%/DM to 85.16%/DM, also recovery enhanced from 53.18% to 68.94%. Other pretreatments used in this study provided less success in enhancing betaine separation and demand additional studies. Further development could be directed towards investigation of betaine separation and recovery by using different centrifugal force.

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betaine from *Beta vulgaris* samples by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 409, 5133-5141.

УТИЦАЈ РАЗЛИЧИТИХ ЕФЕКТА ПРЕДТРЕТМАНА НА УСПЕШНОСТ СЕПАРАЦИЈЕ БЕТАИНА

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Сажетак: Меласа шећерне репе представља најзначајнији споредни производ технологије шећера који се због свог високовредног састава може валоризовати на различите начине. Стандардни састав меласе шећерне репе представљају шећери, нешећерне компоненте и вода. У меласи шећерне репе је обично присутно 3 до 8% бетаина рачунато на садржај суве материје (СМ). Бетаин представља значајну нешећерну компоненту која се не елиминиса током процеса производње шећера, него се акумулира у меласи. Фракција богата бетаином се добија индустријским хроматографским поступком.

Циљ овог рада је да се повећа удео бетаина у бетаинској фракцији и минимизује присуство осталих нечистоћа применом различитих предтретмана пре финалне сепарације. Састав фракција одређен је применом аналитичке технике HPLC, а квантификација је извршена применом калибрационе криве добијене помоћу стандарда бетаина. Сепаративне технике коришћене у овом раду су: (I) сепаративни метод без предтретмана (II) сепарација са претходном ацидификацијом-подешавање рН вредности узорака и (III) сепарација са алкохолном ферментацијом као предтретманом. Удео бетаина у полазном узорку је 62.76%СМ. Највеће повећање удела бетаина је постигнуто применом сепаративне технике без икаквог претходног третмана, где је повећан удео бетаина на 85.16%СМ. Ацидификација као предтретман центрифугирању резултовала је повећањем удела бетаина на 67.68%СМ, док применом алкохолне ферментације као предтретмана центрифугирању није значајно промењен удео бетаина у узорку.

Кључне речи: меласа шећерне репе, бетаинска фракција, ацидификација, алкохолна ферментација, технике сепарације, HPLC

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