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EXTRACTION AND DETERMINATION OF FREE AMINOACIDS AND AMIDS IN SUGAR BEETS

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Studies concerning different methods of extraction of aminoacids from sugar beets were carreid out. The aminoacids, including asparagine and glutamie were separated and determined in an automatic analyzer. Correction coefficients for each method were arrived at. Norleucine was used as the internal standard.

Free aminoacids and amids in sugar beets almost entirely pass into diffusion juices during extraction of cossettes and are not removed in the course of the juice purification. Therefore these compounds are regarded as "noxious" in the sugar-beet technology because they have multidirectional, negative effects on juice quality, including forming of colour compounds, unnecessary lowering of alkalinity and building of molasses [2, 3, 5]. The results of determination of aminoacids and amids are used directly for qualitative assessment of sugar industry raw materials and indirectly for forecasting of the most fit techniques of processing the materials into sugar.

The aim of the present experiments was to determine optimum conditions for extracting free aminoacids and amids from sugar beets as well as their determination by means of liquid chromatography [3, 4, 6].

MATERIALS AND METHODS

The materials for the experiment consisted of sugar beet samples from large plantations (the 1976 crop). For the analysis of aminoacids the samples were desintegrad by means of the sugar industry disc cutter. The

pulp was mixed and stored in deep-freeze. The contents of dry matter in the pulp was determined gravimetrically by drying for 3 hours at 105°C.

The content of saccharose was determined polarimetrically using basic lead acetate [7] for clarification.

The extraction was conducted as follows:

A. With 70° ethanol

B. With 70° ethanol in the Soxhlet aparatus; 200 cc, 8 hrs

C. With 2% solution of trichloroacetic acid (TCA)

D. With 2% solution of hydrochloride in 70° ethanol.

In methods from A through D the dry residue was extracted each time. The extracted quantity corresponded to 15 g of the pulp. Except for Method B, the extraction was continued by triple shaking at room temperature; total volume of solvents used each time was 60 cc. The extracts were condensed in a rotary evaporator (pressure — 30 mm Hg) till nearly dry.

E. With water (total volume — 90 cc) by triple mechanical shaking 10 min. each time at room temperature. 15 g pulp was removed; the extracts were put together and filtrated.

The extracts were deproteinized by infusion of 10 cc 10% TCA solution and centrifuging for 10 min. (4500 rpm). The supernatants were supplemented to a given volume with 0.2 N buffer solution with sodium citrate (pH 2.23) — tryptophan and basic aminoacids — and with 0.2 N buffer solution with lithium citrate (pH 2.23) — neutral aminoacids, dicarboxyl aminoacids and their amids. Concentration of the solution prepared for determination of tryptophan and basic aminoacids was four times higher. The pulp of the 1976 beet crop samples was also extracted with the following three methods using norleucine as the internal standard:

F. As given in E, plus addition of water to norleucine — 0.125 μM/cc.

G. As given in E, and with substitution of water by 0.1 N of the buffer solution with lithium citrate (pH 1.5). The buffer solution contained 0.125 μM/cc norleucine.

H. With 0.1 N buffer solution with lithium citrate (pH 1.5) in a digestion vessel [3, 7]. 26 g pulp and 178.2 cc buffer solution with 0.25 μM/cc norleucine were used.

Digestion was carried out for 30 min at 75°C. After cooling down to room temperature the mixture was filtrated. The filtrate was deproteinized using a contrifuge for 40 min. at 73,000 g. Clarified supernatant was diluted with 0.1 N buffer solution with lithium citrate (pH 2.23) in ratio of 1:1 (v/v). The analysis of aminoacids was conducted in an automatic liquid chromatograph 'Jeolco' Type JLC-6 AH with two columns and two developing systems: buffer solutions with lithium citrate (column 50×0.8 cm) and buffer solutions with sodium citrate (column 15×0.8 cm).

The results of separation of aminoacids, including asparagine and glutamine, were the following:

1. Tryptophan and basic aminoacids:
— stationary phase: sulphonated spherical resin, LCR-2
— mobile phase: 0.2 buffer solution with sodium citrate (pH 5.29 ±0.01
— eluate flow velocity: 1.5 cc/min.
— ninhydrine reagent flow velocity: 0.56 cc/min.
— column temp. and pressure: 65°C, 10-12 kG/sq·cm
— through-flow cell: 2 mm
— recorder tape speed: 120 mm/h
2. Neutral and dicarboxyl aminoacids and their amids:
— stationary phase: sulphonated spherical resin, LCR-2 mobile phase: buffer solutions with lithium citrate

I 0.25 N pH 2.78 ±0.01
II 0.3 N pH 3.15 ±0.01
III 0.3 N pH 3.92 ±0.01

— eluates change: I $\xrightarrow[110\ \text{min.}]{}$ II; II $\xrightarrow[190\ \text{min.}]{}$ III
— eluates flow velocity: 0.84 cc/min
— ninhydrine reagent flow velocity: 0.56 cc/min
— column temp. change and pressure: 36° $\xrightarrow[65\ \text{min.}]{}$ 60°, 20-22 kG/sq·cm

The other conditions same as for tryptophan and basic aminoacids. Proline was determined at a 440 nm wavelength while other aminoacids — at 570 nm.

RESULTS AND DISCUSSION

Conditions of extraction of free aminoacids from sugar beets were analyzed. Five methods of extraction were applied: 70° ethanol (A), 70° ethanol in the Soxhlet apparatus (B), 2% solution of TCA (C), 2% solution of HCl in 70° ethanol (D) and water (E). Five parallel extractions were performed with each method. B, C and D do not provide positive results on account of a too low degree of extraction of nearly all aminoacids. Particularly small values were obtained for dicarboxyl acids and their amids. Moreover, asparagine and glutamic acid as well as glutamine separated from the pulp with Method D formed broad mixed bands on the chromatograph. It can be surmised that this fact is due to extracting of some ninhydrine-positive bases which migrated with glutamic acid, asparagine and glutamine.

In comparison with B, C and D, methods A and E gave satisfactory results on extraction of aminoacids. In the latter the values arrived at were slightly higher.

As concerns Method A, next to the aminoacids listed in Table, ten ninhydrine-positive compounds were also found. Nine of them migrated on

the chromatogram before aspartic acid. The method can be used for analysis of the contents of other compounds in sugar beets which react with ninhydrine. In the context of analyses regarding "noxious nitrogen" in sugar beets the identification of these compounds and their significance was to extremely great interest.

Table 1. Aminoacids in the analyzed sugar beets.
The analytical extract was produced in three ways involving application of the internal standard:
F — water extract
G — buffer extract
H — buffer extract based on digestion method

Aminoacid	Type of extract								
	F			G			H		
	$\bar{x}$	S	V	$\bar{x}$	S	V	$\bar{x}$	S	V
Aspartic acid	1.588	0.016	1.01	1.622	0.027	1.66	1.390	0.033	2.37
Threonine	0.490	0.012	2.45	0.537	0.039	7.26	0.491	0.021	4.28
Serine	1.159	0.013	1.12	1.186	0.022	1.85	1.063	0.054	5.08
Asparagine	1.856	0.037	1.99	1.891	0.058	3.07	1.643	0.070	4.26
Glutamic acid	1.034	0.062	5.99	1.100	0.066	6.00	0.811	0.018	2.22
Glutamine	19.855	0.340	1.71	20.694	0.720	3.48	20.123	0.790	3.93
Proline	traces			traces			traces		
Glycine	0.217	0.017	7.83	0.226	0.019	8.41	0.196	0.014	7.14
Alanine	0.921	0.038	4.13	0.952	0.043	4.52	0.880	0.031	3.52
Alpha-aminobutyric acid	0.189	0.018	9.52	0.197	0.019	9.64	0.121	0.011	9.09
Valine	0.299	0.027	9.03	0.315	0.019	6.03	0.302	0.016	5.30
Cystine	traces			traces			traces		
Methionine	0.090	0.011	12.22	0.099	0.006	6.06	0.090	0.002	2.22
Isoleucine	0.665	0.033	4.96	0.691	0.024	3.47	0.674	0.017	2.52
Leucine	0.696	0.010	1.44	0.727	0.021	2.89	0.675	0.028	4.15
Tyrosine	absent			absent			absent		
Phenyloalanine	0.078	0.008	10.26	0.079	0.005	6.33	0.077	0.006	7.79
Tryptophane	0.172	0.008	4.65	0.193	0.011	5.70	0.179	0.011	6.15
Lysine	0.171	0.009	5.26	0.183	0.016	8.74	0.174	0.009	5.17
Histidine	0.064	0.012	18.75	0.074	0.012	16.22	0.072	0.006	8.33
Arginine	0.144	0.007	4.86	0.150	0.011	7.33	0.141	0.009	6.38
	29.688			30.916			29.102		

$\bar{x}$ — average contents of aminoacids from 10 determinations (mM/100gS)
S — mean deviation (mM/100gS)
V — variability coefficient (%)
Other data on the analyzed beets (23):
Polarization 12.90%; total nitrogen 144.29 mval/100gS; alpha-aminoacid nitrogen — 32.14 mval/100 gS

Extraction of aminoacids from sugar beet pulp with the use of norleucine as the internal standard was also performed. Three methods of extraction were applied: water (F); with buffer solution of lithium citrate (G) and with buffer solution of lithium citrate in a digestion vessel (H).

The values obtained for particular aminoacids and presented in Table approximate one another in the three methods. It must be stressed that in the extract obtained with the buffer solution of lithium citrate (G) the quantity of aminoacids reached the highest level. However, the popularity of Method H in sugar processing laboratories accounts for the fact that it can also be applied to determination of aminoacids in sugar beets.

The dispersion of results from the extraction methods was characterized by calculation of the standard deviation and the variability coefficients. The values were exppressed in mM aminoacid per 100 g saccharose.

Method F rendered the S values within the range from $6.7 \cdot 10^{-3}$ to $6.2 \cdot 10^{-2}$ for most of the aminoacids (for Gln $3.4 \cdot 10^{-1}$); hence the precision of Method F expressed as 2 S fluctuates, depending on a given aminoacid between $13.4 \cdot 10^{-3}$ to $12.4 \cdot 10^{-2}$ mM (for Gln $6.8 \cdot 10^{-1}$).

In Method G the S values vary between $5.4 \cdot 10^{-3}$ and $6.6 \cdot 10^{-2}$ for most of the aminoacids (for Gln $7.2 \cdot 10^{-1}$); hence the extremal values of the precision of Method G are from $10.8 \cdot 10^{-3}$ to $13.2 \cdot 10^{-2}$ (for Gln $14.4 \cdot 10^{-1}$).

The analogous values for Method H are: $5.5 \cdot 10^{-3}$ to $7.0 \cdot 10^{-2}$ (for Gln $7.9 \cdot 10^{-1}$) while the 2S values are: $11.0 \cdot 10^{-3}$ to $1.4 \cdot 10^{-2}$ (for Gln $15.8 \cdot 10^{-1}$).

The different values for glutamine in terms of the standard deviation and precision, in all the three methods, are due to its high level in the pulp. In Methods F, G and H the S values for particular aminoacids are of the same order, although for Method F they were calculated on the basis of two times lower number of the degrees of freedom.

Correction coefficients were calculated for every method of extraction using the internal standard. Their values for F, G and H methods, respectively, are: 1.0788, 1.0731, 1.0899. 21 aminoacids in sugar-beet pulp were determined, including dicarboxyl aminoacids and their amids. The overall time of the analyses was 5.5 hrs, what constituted a considerable reduction in comparison with the data reported in the literature on the subject [1, 6].

CONCLUSSIONS

1. The most useful method for determination of free aminoacids extracted from sugar-beet pulp is the G method utilizing buffer solution of lithium citrate (pH 1.5).

2. Methods F, G, H can be used for extraction of free aminoacids from sugar-beet pulp provided that appropriate corection coefficients are taken into account.

3. The above mentioned conditions for chromatographic work in an automatic analyzer make it possible to separate 21 aminoacids within 5.5 hrs, including asparagine and glutamine.

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EKSTRAKCJA I OZNACZANIE WOLNYCH AMINOKWASÓW I AMIDÓW W BURAKACH CUKROWYCH

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Streszczenie

Celem badań było opracowanie i wybór sposobu ekstrakcji i oznaczania wolnych aminokwasów i amidów zawartych w burakach cukrowych metodą chromatografii cieczowej. Ekstrakcję aminokwasów z miazgi uzyskanej z buraków cukrowych prowadzono następującymi rozpuszczalnikami: 70° etanolem, 2% kwasem trójchlorooctowym, 2% chlorowodorem w 70° etanolu, wodą z dodatkiem norleucyny, 0.1 N roztworem buforowym z cytrynianem litowym o pH 1.5 z dodatkiem norleucyny. W badaniach używano 15 g miazgi i 90 cm^3 rozpuszczalnika. W przypadku roztworu buforowego z dodatkiem norleucyny przeprowadzono ekstrakcję stosując powszechnie używane w przemyśle cukrowniczym do ekstrakcji cukru stężenie miazgi 26 g w 178.2 cm^2 roztworu buforowego oraz wykorzystano tu także cukrownicze naczynko digestyjne. Stwierdzono, że najprzydatniejszy do oznaczania aminokwasów jest sposób z zastosowaniem 0.1 N roztworu buforowego z cytrynianem litowym w naczynku digestyjnym. Dla metod ekstrakcji, w których był stosowany dodatek wzorca wewnętrznego norleucyny, wyliczono współczynniki korekcyjne. Zaproponowane warunki chromatografowania umożliwiły oznaczenia 19 aminokwasów i dwóch amidów w ciągu 5.5 h.

Do analiz stosowano automatyczny chromatograf cieczowy firmy „Jeolco" typ JLC-6 AH wyposażony w dwie kolumny i dwa układy rozwijające roztwór buforowy z cytrynianem litowym i sodowym.