AMINO ACIDS

Content of Sugar Beet Processing Juices

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The amino acids in sugar beets are included in the so-called "harmful" nitrogen that is a factor in the formation of molasses in the production of beet sugar. Information on the identity, amounts, and changes in amino acids in sugar-beet processing liquors is important in the evaluation of purification techniques, in the utilization of by-products, and in following the biochemical changes in beets. Paper chromatography has been utilized to identify the amino acids present in beet juices. Microbiological methods proved time-consuming; hence a quantitative paper chromatographic method was adapted for the purpose. Use is made of "reflectance density" of amino acid spots developed with ninhydrin. As many as 18 amino acids have been indicated in certain beet juice samples by paper chromatography. There is a variation in the number and amount of amino acids in beets from different parts of the country. During processing, including diffusion, glutamine is converted to pyrrolidone carboxylic acid, part of the tyrosine is oxidized, and threonine and serine decrease. The decrease in the amount of threonine and serine at pH 11 is the result of a reversal of aldol condensation rather than the β-elimination encountered at higher concentrations of alkali.

The formation of molasses during production of beet sugar results from interference of the impurities with the crystallization of sucrose, probably by decreasing the activity of sucrose (*12*). As a rough rule each gram of impurity causes 1.5 grams of sucrose to enter molasses. Of the organic compounds concerned, amino acids, with betaine, are the worst offenders because of their abundance and their charge effects. They also form complexes with calcium ion, which increase the amount of lime used during purification of beet juices in the factory and they act as buffers to influence the efficiency of the liming operation (*5*). The identification and determination of these amino acids, therefore, are significant to the evaluation of purification techniques. Such information is also necessary to evaluate better the nutrient quality of molasses and various factory waste waters, to aid in finding the causative agents of browning of massecuites, and to establish the presence of compounds, such as glutamic acid, which might have value as by-products.

Recent work (*2, 3, 4, 6, 7, 10, 11, 13*) has tremendously increased the knowledge of the amino acid content of sugar beet juices and molasses. The work reported summarizes some of the current findings obtained at this laboratory on the amino acid content of beet diffusion juice obtained from different parts of this country. Included are a description of a modified quantitative paper chromatographic technique and a discussion of some of the changes amino acids undergo as a result of present processing procedures.

Preparation of Samples

Sugar beet cossettes were collected by the chemist at the factory and immediately sterilized in 5 volumes of boiling isopropyl alcohol. Factory diffusion juice was obtained from similar cossettes, concentrated in vacuo to 70% solids, and covered with toluene. On receipt at the laboratory, the samples were stored at 3° C. Samples of molasses, collected at the same time, were also stored at 3° C. under toluene.

Alcohol was removed from the cossettes by evaporation: the cossettes were then extracted twice for 30 minutes with boiling water. To provide a reference concentration for comparative purposes, the total solids were measured in a refractometer and most results are reported as milligrams per liter at 10% rds (refractometric dry solids).

In some cases the total sugar was measured and the results were calculated to a 10% total sugar basis.

Each juice was diluted approximately 10% solids and filtered through coarse filter aid, and an aliquot was passed through a strong cation exchanger. In a typical example, 200 ml. of diffusion juice (10.8% rds) was passed through a 40 × 1.6 cm. column of 60- to 80-mesh resin. The flow rate was 40

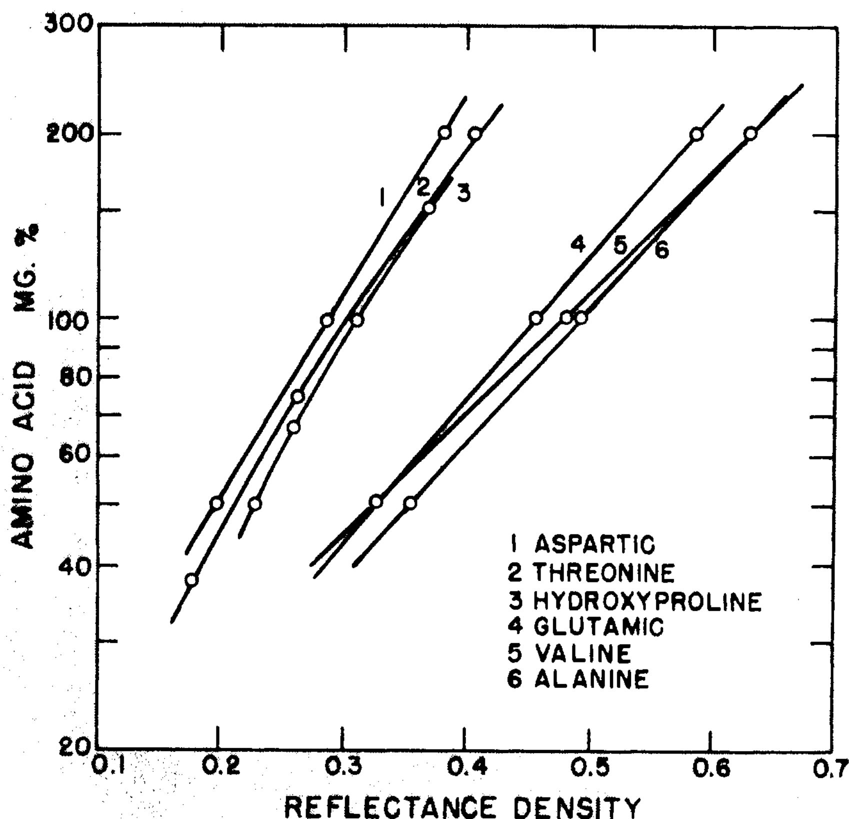

Figure 1. Typical standard curves obtained by plotting log of amino acid concentration against reflectance density

ml. per hour. The resin was washed with 200 ml. of water. The load effluent and washings were separately concentrated tenfold. The concentrates were tested for amino acids by paper chromatography. No amino acids were found. The amino acids were eluted with 3 N aqueous ammonia flowing at the rate of 15 ml. per hour. The first 12 fractions (15 ml. each) of eluate were free of amino acids. The next 8 fractions contained all the amino acids as shown by chromatography. Practically all the acids were concentrated in three 15-ml. fractions (Nos. 13 to 15). Fractions 12 to 23 inclusive were combined and concentrated to 20 ml. to obtain a concentration tenfold greater than the original.

During the fractionation and subsequent storage the samples were preserved with thymol and phenyl mercuric nitrate.

Table I. Composition of Standard Amino Acid Solutions

(Mg. %)

Amino Acid	*I*	*II*	*III*
Aspartic	200	100	50
Glutamic	200	100	50
Serine	150	75	37.5
Glycine	100	50	25
Threonine	100	50	25
Alanine	200	100	50
Valine	150	75	37.5
Leucine	150	75	37.5
γ-Aminobutyric	200	100	50

Analysis by Paper Chromatography

The number and relative amounts of amino acids in the various juices were established by two-dimensional papergrams. Phenol (400 ml.) and water (100 ml.) containing 200 mg. of 8-quinolinol was the developing solvent in the one direction, and 2,6-lutidine (275 ml.), absolute ethanol (100 ml.), water (25 ml.), and diethylamine (5 ml.) was the solvent for the other direction. The technique was essentially that given by Block, Le Strange, and Zweig (*1*). Because no advantage was gained through equilibration of the papers over the solvents or over water, which increased the moisture content to 30%, the papers were employed in the air-dry condition and contained about 3% moisture at the relative humidities existing in this laboratory. Six drops of each solution were superimposed on each paper, so that the concentration of amino acids tested was 60 to 90 times greater than that in the original solution based on a 10% rds basis. The developed chromatograms were dipped into a 0.5% solution of ninhydrin in *n*-butyl alcohol. The standard solutions were made up to contain the amino acids as given in Table I.

For quantitative work one-dimensional papergrams were run by the technique given by Block *et al.* (*1*), with the solvents given above. The lutidine solvent was used primarily for the separation and determination of "leucines," valine, threonine, and as a first approximation of γ-aminobutyric acid, while the phenol solvent was used for the remaining amino acids. Three separate 5-μl. spots of each solution were placed 2 cm. apart on sheets of Whatman No. 1 or 4 or S & S No. 507. Similar spots of each standard were also placed on the paper. Paper 507, although slower in development, gave somewhat sharper spots. Generally, the solvents were allowed to ascend at least 25 cm. above the origin on the paper. After drying overnight at room temperature, the papers were dipped into the ninhydrin solution and allowed to stand overnight for color development. Background color was generally light and uniform.

As a further check, two-dimensional chromatograms were run with 2-μl. spots. In these cases the unknown was placed on one paper (20 × 20 cm.) and the three standards on three other papers. All four papers were run concomitantly in the same jar. Two-dimensional chromatograms permit better separation of γ-aminobutyric acid, glycine, and alanine from interfering amino acids. This technique appears promising for quantitative amino acid determinations when used in connection with reflectance density measurements and is under further examination.

Hydroxyproline was determined on chromatograms developed in *n*-butyl alcohol (250 ml.), water (250 ml.), and acetic acid (60 ml.) (*1*). A solution of 0.4% isatin in *n*-butyl alcohol containing 4% acetic acid was the coloring agent. One amino acid, as yet unidentified, gave the same color as the prolines but did not interfere. The standards in this case contained 200, 100, and 50 mg. % of proline and hydroxyproline; although no proline has been found by means of paper chromatography, microbiological assay showed its presence.

Effect of variation in the optical density of No. 1 paper was avoided by measuring the reflectance of light from the paper by means of a Photovolt reflectance and densitometer unit (No. 5013). A 570 mμ filter was placed ahead of the search unit for ninhydrin colored spots and a 600-mμ filter for the isatin spots. The log concentration of the amino acids was plotted against the average maximum "reflectance density" of the three spots. As shown in Figure 1, the curves were usually straight lines. Overlapping was serious only at the higher concentrations. This disadvantage is reduced by using 2-μl. spots for this type of work. Spots of 1-μl. size require higher concentrations of amino acid and are less satisfactory.

Amino Acid Content of Beet Juices

Table II summarizes the results obtained from the chromatograms, includ-

ing one series of microbiological assays for comparison. The results obtained by both methods are in good agreement when it is considered that they are subject to variations as great as 10%. Only the determinations of glutamic acid differ seriously. This may be the result of hydrolysis of pyrrolidone carboxylic acid (PCA) during adsorption or elution from the resins or during the subsequent evaporation. D-glutamic acid may have formed which would not be utilized by the *L. arabinosus*.

The concentrations of amino acids in juices collected in different factories varied considerably, but this is to be expected, considering the marked variation in agronomic factors: climate, soil, fertilization, irrigation, lighting conditions, and varieties of beets.

Aspartic acid, glutamic acid, threonine and γ-aminobutyric acid vary more than the other amino acids measured. One amino acid, in concentrations comparable to valine, and three acids in lower concentrations have not yet been identified. By plotting the R_f's of amino acids in phenol against the reciprocal of the number of carbon atoms in the chain, as shown in Figure 2, a series of straight lines is obtained. By use of this plot and those of Polson (*8*), three of the unknowns were tentatively identified as β-alanine, lysine, and citrulline. Histidine has not been found.

The chromatographic method as given should prove valuable to the agronomist when following certain genetic characteristics of beets or the effect of fertilization. Similar benefits should accrue for the biochemist studying the changes occurring during growth and maturation of homozygous beets. Similarly, the glutamic acid manufacturer can readily determine the glutamic acid content of the Steffens waste waters after hydrolysis of the pyrrolidone carboxylic acid.

Recent results (*2, 3, 4, 6, 7, 13*) obtained in other laboratories show that beets grown in other parts of the world are qualitatively similar in composition to those grown in this country.

Table II. Amino Acid Content of Sugar Beet Diffusion Juice from Different Areas

(Concentrations in mg./l. at 10% rds)

Amino Acid	Manteca, Calif. A[a]	Manteca, Calif. B[a]	Rupert, Ida., A	Sidney, Mont., A	Carrolton, Mich., A	Moorhead, Minn., A	Toppenish, Wash., A
Glutamic acid	158	106	60	80	140	160	350
Aspartic acid	123	132	90	100	70	140	200
Serine	79	62	35	60	35	50	..
Glycine[b] } Asparagine[b] }	13	13[b]	10	10	10	10	15
Alanine	75	62	35	40	40	40	40
Threonine	26	35	15	15	10	?	25
Hydroxyproline	13	..	15	25	15	..	<5
Tyrosine	Present	11	..	..	..	Present	Present
Valine	44	35	30	30	20	30	25
"Leucines"	88	88	50	80	40	90	80
Phenylalanine	Present	7	..	..	Present	..	Present
γ-Aminobutyric acid	60	..	90	150	200	90	100

[a] A, chromatographic analysis. B, microbiological assays. Latter also showed arginine 2, histidine 4, lysine 9, and methionine 17 mg./l.
[b] As glycine. Microbiological assay does not include asparagine.

Table III. Changes in Concentrations of Amino Acids in Beet Processing Juices from Manteca, Calif.

(Expressed in mg./l. on basis of 10% total sugar)

Amino Acid	Cossette Juice	Diffusion Juice	Molasses[a]
Aspartic acid	150	150	500
Glutamic acid	100	180	630
Glutamine[b]	970	Near 0	Near 0
Pyrrolidone carboxylic acid	0	970	5000
Alanine	60	80	400
Asparagine	Present	Present	Near 0
Glycine	15	15	170
Serine	80	90	230
Threonine	35	80	0
Valine	45	50	210
Tyrosine	70	10	..
Leucines	105	100	660
Unknown	..	Present	Absent
Unknown	..	Present	Present

[a] Molasses formed after removal of about 85% of sugar, therefore amino acid concentrations should increase by 5- to 6-fold.
[b] Glutamine converted to pyrrolidone carboxylic acid, then to glutamic acid, and determined as such.

Table IV. Alkaline Degradation of Hydroxy Amino Acids

(Concentrations in millimoles/l.)

Amino Acid	Condition	Concentration before Heating	Concentration after Heating
D,L-threonine	pH 11 lime	42	Threonine 21 Glycine 17
L-threonine	pH 11 lime	21	Threonine 2 Glycine 16
L-threonine	$3N$ NH_3	21	Threonine 21
L-serine	pH 11 lime	24	Serine 12 Glycine Ca 10
γ-aminobutyric	pH 11 lime	50	Alanine Ca 4 γ-Aminobutyric 17

Changes in Amino Acids During Processing

Although amino acids go through the purification process now used in beet factories and are thus considered melassigenic, some changes occur, as shown in Table III. The most pronounced change is the well-known conversion of glutamine to pyrrolidone carboxylic acid, which begins to occur during heat treatment beets receive while in the diffuser, but is greatly accelerated during liming and carbonation. Asparagine apparently hydrolyzes during liming, probably to aspartic acid and ammonia. Tyrosine shows a decrease between cossette juice and the diffusion juice, probably because of oxidation during diffusion. The amount of threonine and serine decreases during the liming operation, while glycine seems to increase.

The possibility that alkaline degradation (*12*) of the hydroxy amino acids occurs despite the mild alkaline treatment during liming appeared worth checking. Solutions containing 21 to 42 millimoles per liter of threonine or serine were adjusted to pH 11 with lime and refluxed for 24 hours. The results obtained by means of quantitative chromatography, given in Table IV, show that most of the L-threonine and L-serine that are degraded undergo a reverse aldol condensation to yield glycine. The presence of acetaldehyde or formaldehyde in the heated solutions was proved by reaction with *p*-hydroxydiphenyl and 2,7-dihydroxynaphthalene, respectively. The presence of a spot at the same R_f as alanine agrees with the findings of

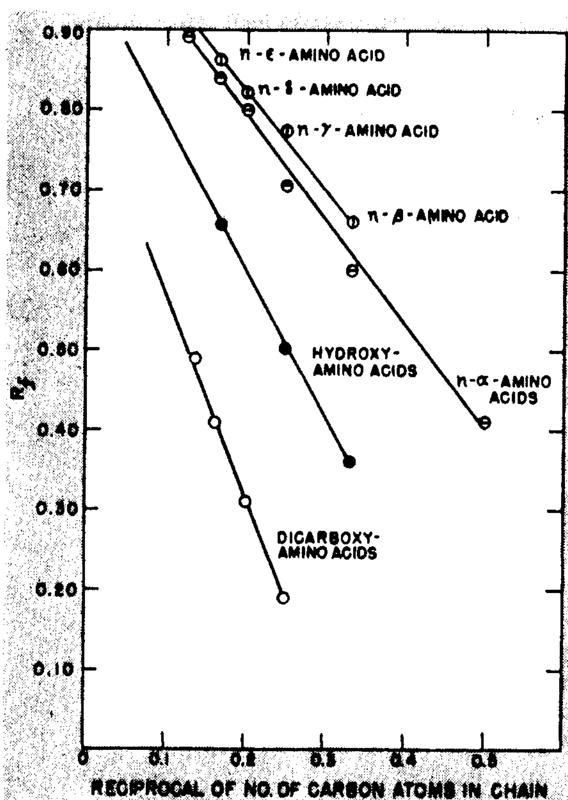

Figure 2. R_f in phenol plotted against reciprocal of number of carbon atoms in amino acid chain

Wieland and Wirth (*14*). Judging from the difference in the rate of degradation of D,L-threonine and L-threonine, there is a structural factor controlling the alkaline degradation of threonine. These reactions appear worthy of further attention.

In addition to loss in threonine and serine during liming, it seemed that one of the unidentified amino acids which has an R_f near that of β-alanine disappeared. A test to determine whether the alanine undergoes β-elimination was negative. Ornithine has been investigated and found to form two new ninhydrin reacting compounds which have not been identified, although one is probably a pyrrolidone complex. Similarly, γ-aminobutyric acid diminishes in quantity on heating in mild alkaline solutions.

It is apparent that alkaline hydrolysis of protein or alkaline treatment of amino acids is likely to cause marked changes among the amino acids and such treatment should be checked for its effect before conclusions are drawn from subsequent analyses. Removal of ammonia by rapid evaporation apparently has very little effect on threonine, as shown in Table IV.

Acknowledgment

The authors are grateful for the generous cooperation of the beet sugar companies in supplying the samples used in this work and to R. J. Brown, Great Western Sugar Co., for a sample of γ-aminobutyric acid. They are indebted to Neva Snell and Roberta Erlandsen for the microbiological assays, to A. Bevenue for the total sugar analyses, and to Howard Walker, Jr., for valuable suggestions concerning the alkaline degradation of amino acids.

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Received for review March 6, 1953. Accepted April 21, 1953. Presented before the Division of Carbohydrate Chemistry at the 123rd Meeting of the AMERICAN CHEMICAL SOCIETY, *Los Angeles, Calif.*

GREEN LEMON MOLD

Gaseous Emanation Products

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Common green lemon mold (*Penicillium digitatum*) produces ethylene, which powerfully stimulates lemon respiration. An irradiated mutant mold failed to stimulate lemon respiration. The chemical differences of the emanations from the two molds were investigated. Vapors from moldy lemons were collected by activated carbon adsorption, desorbed in vacuo, and analyzed by mass and infrared spectra. The mutant mold produced less total volatile material than the nonmutant mold, and there were significant qualitative chemical differences between the two emanations. The stimulating effect of *Penicillium digitatum* on respiration of healthy lemons can be reduced by purifying the air contaminated by the mold gases with brominated activated carbon. An alternative method consists of simultaneous diffusion of small concentrations of bromine into the storage space and recirculation of the storage air through unbrominated carbon.

THE GASEOUS EMANATIONS from common green lemon mold (*Penicillium digitatum*) have been shown (*3*) to contain a powerful stimulant for lemon respiration, probably ethylene. Figure 1, taken from Biale and Shepherd (*3*), illustrates the course of this stimulation. The evidence that the stimulating gas is, in fact, ethylene includes the following: The stimulation of lemon respiration by pure ethylene is similar to that produced by gaseous emanations from the mold;