UDC 547.466.2:664.12

103. Takeo Tsukamoto, Tetsuya Komori, and Nadao Kinoshita : Microanalysis of Amino Acids. II.[1] On the Amino Acid Content of Beet Molasses from Hokkaido.

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The quantitative analysis and separation of amino acids by ion-exchange chromatography, column partition chromatography, column electrophoresis, and paper partition chromatography have been carried out by many workers. In our laboratory, paper partition chromatography is used for its relative simplicity of procedure in the separation of amino acids. As has been reported previously,[1] ninhydrin was successfully improved as a reagent and the conditions required for photometric analysis of amino acids determined. Later research, in which ion-exchange chromatography was also applied, has resulted in a successful analysis and separation, in a satisfactory quantity, of the amino acid content of beet molasses from Hokkaido, as will be described herein.

The main components of beet molasses and of cane molasses were dealt with by Wohryzek.[2] More recently, Cole, *et al.*[3] reported that ion exchange resin was available in the refining of sugar. Ion exchange resin has come to be used later in reëxamining the composition of amino acids and betaine. However, amino acid has usually been analyzed qualitatively[4~8] and not quantitatively except by Strocchi[9] who determined glutamic acid and 8 other amino acids quantitatively.

In the methods hitherto used, beet molasses has to be deprived of its sucrose as saccharate by the use of calcium oxide and barium hydroxide before its amino acid content can be examined. However, these methods are unsatisfactory in that the amino acid itself forms a salt and is adsorbed to the calcium and barium saccharate precipitating in a large quantity. In our experiment the error occurring during the procedure was prevented on the principle of group separation, reported by Tiselius,[10] Kunin,[11] and Partridge.[12] A diluted solution of beet molasses is passed through activated carbon and ion exchanger column, the groups of separated, adsorbed amino acids are eluted, and analyzed by one- and two-dimensional paper chromatography and thus amino acids determined are quantitatively separated from sugar and inorganic salts.

The authors are indebted to Mr. M. Shimidzu of Meiji Sugar Mfg. Co., Ltd. for supplying molasses used for the present experiment. The authors wish also to express their thanks to Miss Y. Inoue for technical assistance and to the members of the Microanalytical Room of this Institute for the microweighing.

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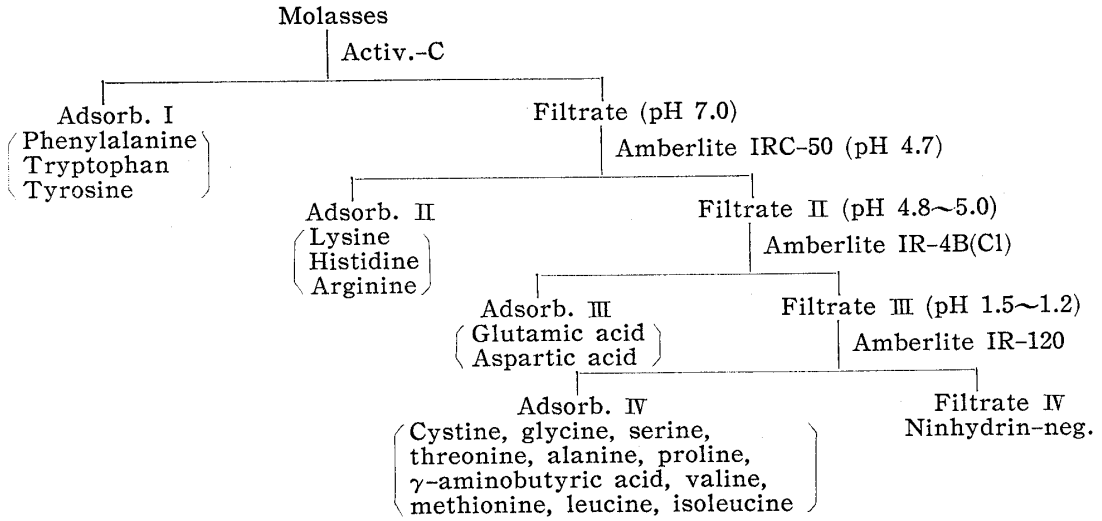
Experimental

Materials used : Beet molasses from Hokkaido (presented by the Meiji Sugar Mfg. Co., Ltd.).

TABLE I. Qualitative Separation



Qualitative Analysis—Molasses (10 g.) is added with distilled water bringing up the total volume to 300 cc. (d.=1.01, pH 6.0~6.2) and filtered through a sintered glass filter No. 3. The filtrate is passed at a rate of 2 cc./min. through a column (1-cm. diam.) with a sintered glass filter No. 3 attached to it. The column contained 1 g. of activated carbon, which has been boiled in 20% AcOH for 1 hr., washed with distilled water, and kept in 5% AcOH. The carbon column is washed first with 5% AcOH (10 cc.) and next with distilled water (50 cc.) and added with 10% NaOH to bring the pH to 7.0 (Filtrate I).

The carbon-adsorbed portion is eluted with 20% AcOH (50 cc.) containing phenol (2.5 g.); the phenol is removed by extraction with ether (20 cc.) and the remainder is concentrated under reduced pressure (Carbon-adsorbed Fraction I).

This adsorbed fraction is subjected to one-dimensional paper chromatography by the ascending method,[13] using BuOH-AcOH-H_2O (4:1:2) as developing solvent, for confirmation of the presence of tyrosine, tryptophan, and phenylalanine (Rf 0.48, 0.55, and 0.62).

Filtrate I is passed at a rate of 2 cc./min. through a column (1.35-cm. diam.) of 30 cc. of Amberlite IRC-50 and buffered with AcOH to pH 4.7. It is next mixed with a solution produced after the washing with 150 cc. of distilled water (Filtrate II). The fraction adsorbed on the Amberlite IRC-50 is eluted with 0.2*N* HCl, added with water, and concentrated again under reduced pressure for removal of HCl (Adsorbed Fraction II). This fraction is treated by the one-dimensional ascending chromatography[14] with the lower layer of the pH 9.3 borate buffer-phenol-*m*-cresol (1 cc.:1 g.:1 g.) as developing solvent, for confirmation of the presence of lysine, histidine, and arginine (Rf 0.22, 0.41, and 0.53).

Amberlite IR-4B (15 cc.) is poured into a column (1.35-cm. diam.) and converted to Cl-form by the addition of 5% HCl.

Through this column Filtrate II, concentrated to some 300 cc. under reduced pressure, is passed at a rate of 1 cc./min.

The effluent is added to the washing (500 cc.) of distilled water and concentrated (Filtrate III). The fraction adsorbed on the Amberlite IR-4B (Cl-form) is eluted with *N* HCl (200 cc.), added with water, and concentrated repeatedly under reduced pressure (Adsorbed Fraction III). This fraction is examined in the same way using a mixture of BuOH-AcOH-H_2O (4 : 1 : 2) as developing solvent for confirmation of the presence of aspartic and glutamic acids (Rf 0.25 and 0.31).

Amberlite IR-120 (30 cc.) is poured into a column and hydrogenated with 10% HCl. Filtrate III is passed through this column at a rate of 1 cc./min., washed with distilled water (300 cc.) which is added to the effluent, and concentrated at pH 7.0 (Filtrate IV). This fraction IV is a sweet-tasting, syrupy substance, negative to the ninhydrin test.

13) The apparatus and methods used for paper chromatography will be described in a forthcoming paper. In this qualitative experiment, filter paper used was Toyo Roshi No. 50 except in the two-dimensional paper chromatography.

14) Filter paper was buffered with pH 9.3 borate buffer.

The portion adsorbed on Amberlite IR-120 is eluted and concentrated under a reduced pressure by the method described above (Adsorbed Fraction IV). This is examined by two-dimensional paper chromatography[13] (Toyo Roshi No. 131) for confirmation of the presence of serine, glycine, alanine, valine, methionine, leucine, isoleucine, threonine, cystine, proline, and γ-aminobutyric acid.

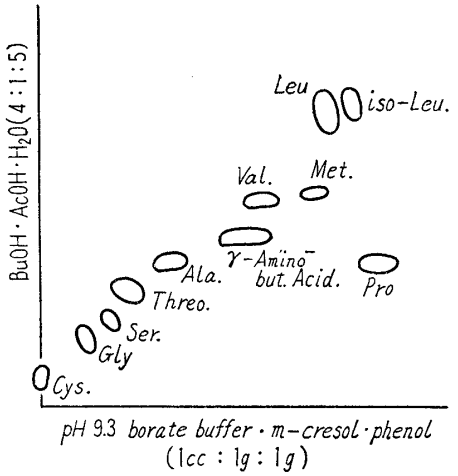
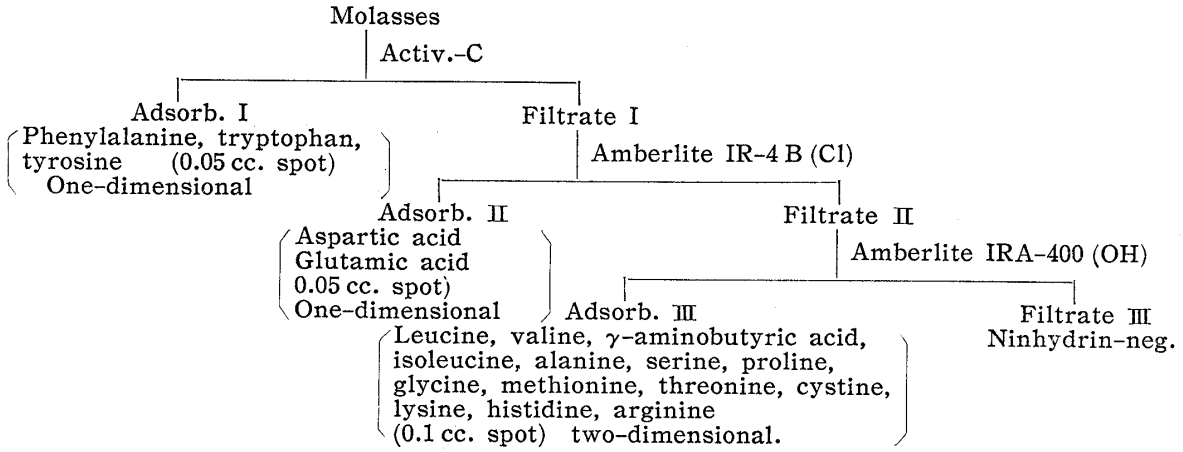


Fig. 1.

Two-dimensional Paper Chromatography of Adsorbed Fraction IV (cf. Table I)

Table II. Quantitative Separation



Quantitative Analysis—Molasses (D_{23} 1.41) (1 g.) is diluted with distilled water (10 cc.) and the diluent is passed at a rate of 0.2 cc./min. through a column (1.2-cm. diam.), of activated carbon (described above). The fraction adsorbed on the carbon is eluted with 20% AcOH solution containing 5% phenol and treated with ether. It is next concentrated cautiously in a glass dish, added with distilled water, concentrated three more times, and diluted with distilled water to a total volume of 1.0 cc. Of this volume, 0.05 cc. is spotted on a piece of filter paper (1.2×40 cm., Toyo Roshi No. 131) by means of a micropipette to be developed by the method described above with BuOH-AcOH-H_2O (4 : 1 : 5 volume ratio). The paper is washed with ether, sprayed lightly with 0.1% EtOH solution of ninhydrin for coloring. The paper is cut off at once and the chromatogram on the paper is extracted with 1 cc. of water for colorimetric determination of aromatic amino acids by the method described in the preceding paper.[1]

The carbon column is washed with 5% AcOH (10 cc.) and some 30 cc. of water. Filtrate I is concentrated to 2 cc. and passed at a rate of 0.5 cc./min. through a column of Amberlite IR-4 B (Cl-form), 8 mm. in inside diameter. Adsorbed fraction II thus obtained is eluted with *N* HCl and concentrated repeatedly with caution in a glass dish. It is next diluted exactly to 1 cc. with distilled water, developed in portions of 0.05 cc. by the one-dimensional paper chromatography, and photometrically determined, as in the case of aromatic amino acid.

The fraction obtained by washing the column with 50 cc. of distilled water is collected and concentrated exactly to 2 cc. (Filtrate II). This filtrate is passed at a rate of 0.5 cc./min. through Amberlite IRA-400 (OH form) (2 cc.) to remove the inorganic salts (Filtrate III).

The fraction adsorbed on Amberlite IRA-400 (adsorbed fraction III) is eluted with 5% AcOH and the amino acids, basic and neutral, are concentrated exactly to 2 cc. and 0.1 cc. of the concentrated part is spotted for two-dimensional paper chromatography. The filter paper is washed with ether and sprayed sparingly, first, with AcOH-EtOH (3:10) (5 cc.) and next with 0.1% EtOH solution (5 cc.)

of ninhydrin and left standing for 2 mins. at 80° for coloring. The chromatogram is identified by comparison with the control, cut off at once, divided into small pieces, and extracted with water (1 cc.) for photometric determination by the procedure described previously.[1] Filtrate III will be found negative to ninhydrin test. The same procedure is repeated with 18 different standard mixtures of amino acids (10 mg. in H_2O) and the amino acid content of the molasses is determined from the equation (1) given below.

Results and Discussions

The amino acid content of the control is shown in Table III. Supposing that the optical density of the standard amino acids, determined on actual measurement,

Table III. Standard Amino Acid (γ/10 cc. H_2O)

Glycine	350	Lysine	760
Alanine	365	Arginine	807
Valine	465	Histidine	860
Leucine	529	Tyrosine	730
Isoleucine	563	Tryptophan	919
Serine	434	Phenylalanine	642
Threonine	468	Glutamic acid	503
Proline	518	Aspartic acid	580
Methionine	632	γ-Aminobutyric acid	446

is Y_I, the whole optical density of molasses, determined from the amino acid component of the molasses, is Y_{II}, and the concentration of the standard amino acids is X_I, the amino acid concentration of the molasses, X_{II}, is determined from the following equation :

$$X_I \frac{Y_{II}}{Y_I} = X_{II} \qquad (1)$$

The amino acid composition of the molasses, determined by equation (1), is given in Table IV. From this amino acid composition of the molasses, the α-amino-N content in 1 g. of the molasses is obtained as 4.25 mg. Quantitative determination in 5 mins. at 756 mm. Hg and at 21°, by the Van Slyke apparatus for use in the determination of α-amino nitrogen, shows that 16.67 mg. of molasses contains 0.122 cc. of amino-N, namely that 1 g. of molasses contains 4.12 mg. of amino-N (average of 5 values). This value, differing 5% or less from 4.25 mg. calculated shows that amino acids

Table IV. Composition of Amino Acid contained in Beet Molasses

Amino acid	Y_I	Y_{II}	mg./g.	%	Amino-N (mg./g.)
Tyrosine	3960	1660	0.3	0.73	0.023
Tryptophan	1980	320	0.2	0.48	0.01
Phenylalanine	5020	740	0.1	0.24	0.01
Glutamic acid	4860	188020	19.4	47.20	1.85
Aspartic acid	1740	33920	11.3	27.50	1.19
Leucine	660	3980	3.2	7.80	0.39
Isoleucine	480	880	1.0	2.43	0.11
Methionine	560	trace	—	—	—
Valine	680	3220	2.1	5.10	0.26
γ-Aminobutyric acid	70	200	1.6	3.90	0.19
Proline	460	320	0.4	0.97	—
Alanine	2880	5440	0.7	1.70	0.11
Threonine	4060	trace	—	—	—
Glycine	8680	3040	0.1	0.24	0.02
Serine	3520	5480	0.7	1.70	0.09
Arginine	4320	trace	—	—	—
Histidine	1580	〃	—	—	—
Lysine	1400	〃	—	—	—
Cystine	—	〃	—	—	—

Y_I : Total value of optical density for standard amino acid.
Y_{II} : Total value of optical density for molasses amino acid.

are separated and analyzed in a larger quantity than by any other method hitherto used when the molasses examined was mixed with sugars, proteins, or rubberized substances. The tryptophan content of molasses has never been mentioned in any literature. The result of paper partition chromatography by the one- and two-dimensional methods and recovery rates will be dealt with on another occasion.

Summary

Nineteen kinds of amino acid contained in beet molasses from Hokkaido were quantitatively separated and analyzed by the new method in which ion exchanger and paper partition chromatography were applied, when the amino acids were mixed with sugars, proteins and other inorganic substances.

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104. Shoji Shibata, Izumi Imaseki, and Mikio Yamazaki: Phytochemical Investigation on Cultivation of Medicinal Plants. XIV*. On the Alkaloid Biogenesis in Ephedra. (4).**

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Earlier papers of this series introduced some new findings on the biosynthesis of ephedrine in Ephedra. ^{15}N-Labeled amino group of phenylalanine, but not alanine, was proved to be incorporated into *l*-ephedrine by one week's cultivation of Ephedra in the Houghland solution added with ^{15}N-labeled amino acids.[1] Therefore, it is quite likely that phenylalanine serves as a precursor of ephedrine. The preceding work also provided an evidence that ^{14}C-methyl group of methionine is transferred into N-methyl group of ephedrine in intact Ephedra plant.[2]

The present experiment has been designed to obtain a precise evidence for the origin of methyl group in the γ-position of the side chain in ephedrine molecule, and ^{14}C-labeled formate was administered to Ephedra plant to investigate the localization of radioactivity in *l*-ephedrine molecule isolated after ten days' cultivation.

Experimental

Plant Material—*Ephedra distachya* L. grown in the Experimental Field for Medicinal Plants attached to Tokyo University Forestry Experimental Station at Tojo, Chiba Pref., was lifted from the ground on May 22, 1957. The hydroponic cultivation of the plant started from the following day employing the Houghland solution added with ^{14}C-labeled formate.

The components of the solution are as follows: KNO_3, $5\times10^{-3}\,M$; KH_2PO_4, $1\times10^{-3}\,M$; $Ca(NO_3)_2$·

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* Part XIII. This Bulletin, **5**, 447(1957).
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