Some Aspects of Solute Concentration and Zone area of the spot for Detection and Quantitation of Amino Acids by Paper Chromatography

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Summary: Detection of amino acids at different concentrations have been carried out. R_f values have been found to change with change of the concentration of amino acids. Quantitative estimation of the separated amino acids from mixture and juices (fruits and vegetables) have been accomplished using area measurement of the spots, both by square-counting and integral evaluation methods. Comparison of zone area with weight of the zone area reveals that the two methods give the same quantitative results.

Introduction

Amino acid analysis covers a wide variety of areas. The important of which are nutritional research, food and beverage industry. In general the samples are protein hydrolysate, biological fluids, fruit juices or free amino acids. Physiologic or free amino acid analysis is important in order to establish a relationship between diet, disease and metabolic condition. Similarly routine amino acid analysis is performed on samples of fruit juices and wines to detect adulteration or the presence of formulation-induced organic by-products. Separation and quantitation of the amino acids demand not only a high chromatographic resolution but reproducibility of the method for quantitative analysis. Until the last decade, liquid chromatographic separations on ion-exchange columns was combined with post-column reaction with ninhydrin to obtain derivatives with absorbance in the visible region [1]. Recent development includes the application of high performance liquid chromatography (HPLC) for plasma amino acids [2] or it is coupled with pre- or post-column derivatisation reaction of amino acids with ortho-phthalaldehyde/2-mercaptoethanol (OPA/2ME) [3], fluorescamine [4], dansyl chloride [5] and phenylisothiocyanate (PITC) [6]. Spectrofluorimetric method [7] following separation by paper chromatography or HPLC has been applied to physiological samples.

Following HPLC, the modern advanced technique, the detection of the compounds by UV or fluorescence method has been made much easier no doubt, but the old fashioned laboratories relying on

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simple technique would like to use preferably the paper or thin-layer chromatography. Simple method for identifying citrus juices [8.9] and amino acids [10] employing paper chromatography are available. The organic acids of various non-citrus juices present as adulterants form characteristic chromatogram and thus they have been identified by paper chromatography [11]. After identification of amino acids their quantitative determination poses a difficult task, especially when the quantity is small. The quantity of nitrogen, found from the analysis of fruit juice, forms the basis for reporting amino acid contents. However, there is one another well established approach to determine the amino acids. The amino acids are expressed as the formol number which is based on the formol titration. The latter is performed potentiometrically [12] to pH 8.5. The formol number is then expressed in terms of mls of N(M) sodium hydroxide per litre.

The present work is intended to improve the procedure for identification and quantitation. Modification of the solvent system, study of the effects of amino acid concentration on the retardation factor (R_f) and measurement of zone area of the chromatographed and developed spots based on square-counting and integral evaluation are the ultimate goal. Application of the analytical procedure will include separation of amino acids from mixtures and juices (fruit and vegetables) and their quantitation.

Results and Discussion

1. Separation of amino acids as a function of solvent

The effect of solvent on the amino acids elution was studied by using solvent systems of desired composition. Preliminary experiments demonstrated that separation of amino acids by paper chromatography occurred using mixture of polar solvents and then a more detailed study was then carried out with various solvents in mixture.

The various solvent-resolution results obtained indicated that the maximum resolution of the amino acids was obtained with solvent systems, *n*-butanol-acetic acid-water (12:3:5) (solvent I) and phenol-water (500 phenol in 125 ml water (solvent II). Nevertheless, the nature of the solvent system greatly influenced the amino acid separation: the more balanced the polarity of the solvent (nonpolar solvent) molecule the higher resolution observed (Table 1). Therefore, solvent I and II were selected for subsequent studies. The amino acid resolution was virtually a failure when using these solvent system with changed composition ratios.

The R_f values in Table 1 provide a better reflection of a successful resolution of components from a mixture of glycine, glutamic acid, alanine, arginine and a number of other amino acids. Results are comparable to R_f values of standard amino acids run individually and separately. However, the R_f values for amino acids in mixture have been found to decrease slightly which may be due to some weak solute - solute interaction.

The eluting process was affected as the solvent system contained more of water. Using the solvent, *n*-butanol - acetic acid - water (5:2:3) (solvent III), there was observed long tailing and band broadening.

2. Influence of amino acid concentration

The concentration of the solute also seems to have some effect on the R_f values.

A few of the selected concentration - R_f graphs obtained are shown in Figs. 1 and 2. As can be seen the R_f values of the amino acids in two different solvents I and II showed a similar behaviour of decreasing R_f values with decrease of amino acid concentration. This is, however, true that change is gradual and noticeable. The explanation for such change may be given that the water soluble amino acids tend to move relatively slowly as the water content in the sample exceeds a certain limit. This, however, cannot be concluded for sure, since the change in R_f values was found not to be alarming even after diluting the amino acid solution to 10^{-4}M (See Table 1).

3. Quantitative estimation

The chromatographic separation was further made more useful by making it quantitative. At pre sent there are number of methods available for quantifying the amino acids such as dissolving of the spots followed by absorptiometry or measuring fluorescence (providing the spraying reagent forms a fluorescent derivative). However, the author preferred to measure the spot area for obtaining the amount of amino acids in a sample.

Table 1: Concentration effect on R_f values

	R_f values							
	Solvent I: BuOH/HOAc/H_2O				Solvent II: PhOH/H_2O			
	Concentration of amino acids				Concentration of amino acids			
Amino acids	1M	0.01M	0.001M	0.0001M	1M	0.001M	0.001M	0.0001M
L-Glycine	0.34	0.31	0.27	0.27	0.80	0.76	0.70	0.65
L-Arginine	0.28	0.23	0.21	0.18	0.64	0.34	0.31	0.28
L-Alanine	0.40	0.38	0.31	0.35	0.62	0.54	0.49	0.43
L-Glutamic acid	0.63	0.60	0.53	0.48	0.33	0.29	0.24	0.21
L-Lysine	0.43	0.20	0.20	0.18	0.58	0.53	0.52	0.49
L-Leucine	0.70	0.65	0.58	0.60	0.82	0.76	0.72	0.69
L-Phenylalanine	0.50	0.37	0.32	0.30	0.86	0.72	0.68	0.61
L-Tyrosine	0.36	0.32	0.24	0.17	0.62	0.55	0.50	0.47
L-Valine	0.55	0.57	0.51	0.44	0.80	0.69	0.64	0.59
L-Aspartic acid	-	-	-	-	0.50	0.46	0.41	0.38
L-Histidine	-	-	-	-	0.85	0.80	0.75	0.69
L-Proline	0.52	0.48	0.43	0.39	0.89	0.79	0.72	0.64
Hydroxoy proline	0.36	0.30	0.26	0.21	0.67	0.59	0.57	0.50

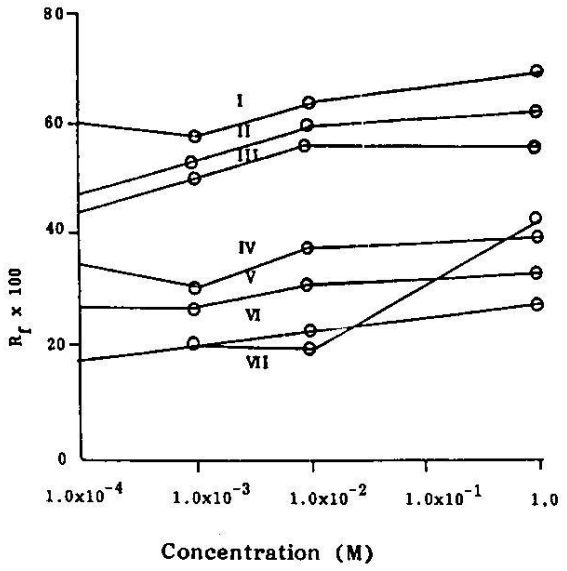

Fig.1: R_f values of amino acids at their different concentrations in solvent system No. 1. I. Leucine, II. glutamic acid, III. Valine, IV. alanine, V. glycine, VI. arginine and VII. lysine. Solvent System No. 1: *n*-butanol: acetic acid: water; ratio: 12:3:5.

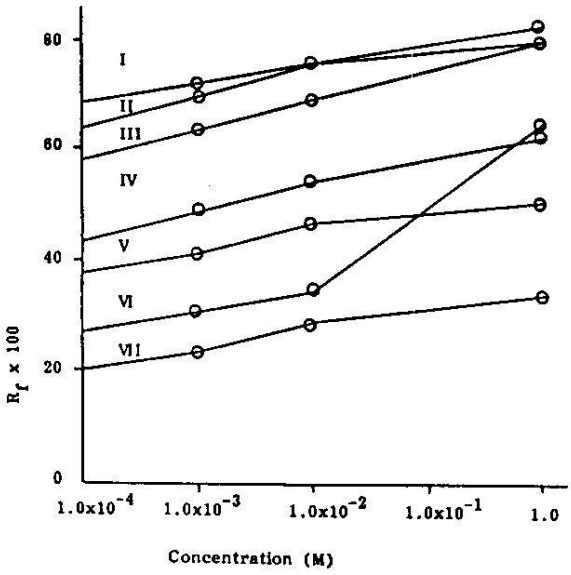

Fig.2: R_f values of amino acids at their different concentrations of molarity in solvent system No. 2. I leucine, II. glycine, III. valine, IV. alanine, V. aspartic acid, VI. arginine and VII. lysine. Solvent System No. 2: Phenol : water (4:1).

(i) Zone area of the spots

In fact there are some striking features in the determination of amino acids by the area measurement method. The accuracy in the measurement depended solely on the methodology. The results presented in Table 2 have been obtained by counting the number of squares after placing the spots on a graph paper [13]. The counting was performed a number of times for the same spot. As can be seen in Fig. 3 the spots were of different shapes and sizes. For those spots which appeared as in Fig. 3(i), they were placed on the graph paper so that a square of measured length was formed alongwith a 3/4th of a circle. The area of the square (= length x breadth) together with the area of the incomplete circle (=3/4 π r^2), where r is the radius of the circle, gave the area of the spots. The area of the spots may also be obtained

Table 2: Relationship between concentration and zone are

	Zone area (Squares)				
	Concentration (M)				
Amino acids	1.0	$1.0x10^{-1}$	$1.0x10^{-2}$	$1.0x10^{-3}$	$1.0x10^{-4}$
Glycine	12.00	9.00	6.00	4.00	3.00
Arginine	42.00	32.00	25.50	17.00	9.00
Alanine	30.00	24.00	17.00	11.00	6.00
Lysine	42.00	34.00	24.20	16.00	7.04
Leucine	40.00	32.00	23.00	16.00	8.00
Proline	43.00	32.00	23.00	18.00	8.00
Valine	31.00	15.00	7.00	5.00	5.25

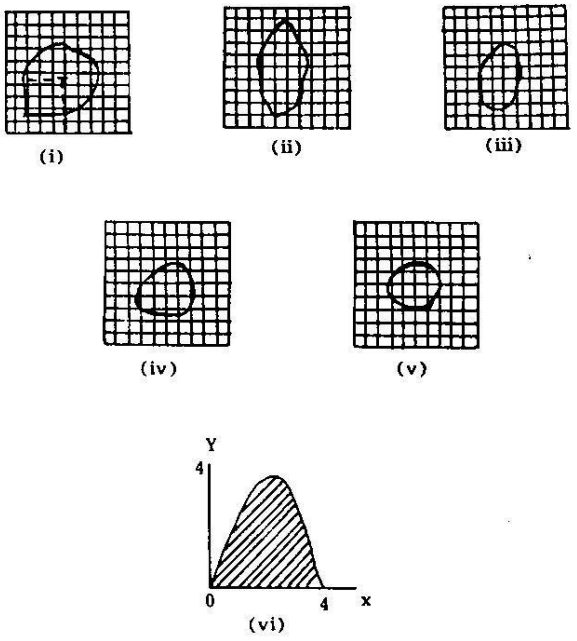

Fig. 3: Different spots of amino acids that appear on the chromatogram after spraying. The shaded area of spot in (vi) respresents only 1/2 of the spot.

by evaluating integral of the shaded portion Fig. 3 (vi) is only 1/2 of the spot and may be assumed to be formed by drawing, on square paper, the graph of $y = x(4-x)$. The shaded area was thus obtained from $\int_0^4 x(4-x)dx$. The exact value obtained by evaluating the integral was used to compare the square-counting method. By comparing the results, it was observed that the square-counting method was not biased at the 1% level using t-test as indicator. However, the method at 5% leave was found to be biased. The weight of the spots are found to increase with increase of zone area. The increase in weight is a function of the size of the spots and is proportional to the quantity of the separated substance. The only problem associated with this is that in the case of smaller spots the weight may not be accurate. However, in the present study the weight has been co-related with the number of squares for comparing the two results. The results have been presented in Table 3. The number of squares was counted on a previously weighed large sheet of paper and the number of squares for the individual spot was then evaluated using the above sheet as reference.

Table 3: Zone area (Squares) by two different methods

Amino acids (10^{-3}M)	Square counting method	weighing method	Difference
Glycine	4	3	1
Arginine	17	17	0
Alanine	11	12	- 1
Lysine	15	13	2
Leucine	16	16	0
Proline	5	3	2

The results are then statistically treated. The measurements are paired i.e. there is one measurement by each method on each specimen and the difference between each pair is calculated. Further, the mean , $\bar{x}\,d = 0.66$, and the standard deviation sd = 1.2, of these differences have been calculated. Adopting the null hypothesis that the methods give the same result, a test is made of whether $\bar{x}d$ differs significantly from zero, using: $t = (\bar{x}d/(sd/\sqrt{n})$ with n-1 degrees of freedom. Thus $t = 0.66/(1.21/\sqrt{6}) = 1.33$. The critical value of [t] for 5 degree of freedom is 2.57 (P=0.05): There is no evidence that the methods give different results.

(ii) Zone area versus concentration relationship

The zone area was found to be dependent upon the concentration , C, of the amino acids.

Fig. 4 represents the relation between log c values of selected amino acids and zone area of the spots. The correlation reflects quantitative application of the procedure. It is seen that there exists a great similarity in the behaviour of the studied amino acids with reference to the area of spots formed on the chromatogram. Arginine, alanine and glycine showed decreasing order of zone area. This trend may be

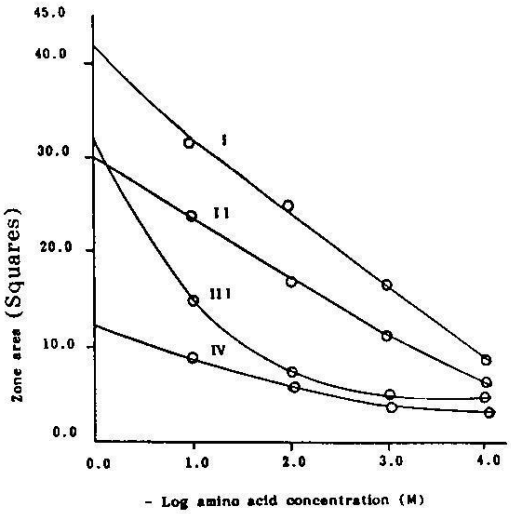

Fig. 4: Changes in zone area as the concentration of the amino acid incrased or decreased. I. arginine, II. alanine, III. valine and IV. glycine.

thought to have some contribution from their own structures. Valine, however, was found to show a little deviation from linearity at lower concentrations. This could be that the solvent-solute interaction may have affected the process but the experimental error cannot be ruled out.

Absorptiometry i.e. measurement of absorbance of the ninhydrin-amino acid derivative in solution [14] at maximum absorbance (340 nm) was carried out for comparison purposes. However, this procedure failed to give satisfactory results and was abandoned. Similarly, the reaction of ortho-phthalaldehyde-2-mercaptoethanol with amino acid which forms isoindole as the fluorescent reaction product could not be followed due to non-availability of fluorimeter. Alternatively, the wavelength of excitation (350 nm) of the isoindole [3] was used for measurement of absorbance. This also did not give promising results as compared to square-counting method.

(iii) Determination of amino acids in juices from fruits and vegetables

The proposed analytical method was applied to determine the content of amino acids in juice from fruits and vegetables (orange, apple, tomatoes and lemon). The results (Table 4) show that the method gave satisfactory results in terms of both qualitative and quantitative. The identification of separated

Table 4: Amino acids in juices from fruits and vegetables

Amino acids	Amounts of amino acids (mg/100 g)			
	Orange	Apple	Lemon	Tomatoes
Glutamic acid	-	5.8	20.5	-
Alanine	15.0	-	-	23.0
Glycine	20.0	-	-	-
Serine	21.0	-	-	-
Proline	-	-	19.7	-
Valine	-	-	-	15.7

amino acids suffered a little set-back due to the R_f values of some amino acids being very close to each other. For this reason only a few selected amino acids were quantified. Otherwise the quantitative determination based on zone are measurement is a practical preposition.

Experimental

A. Experimental

Amino acids such as alanine, glycine, lysine, proline, histidine and tyrosine were obtained from E. Merck. The other amino acids used during the present studies were purchased from B. D. H.

All other reagents used were of AnalaR grade.

B. Preparation of solutions of amino acids and reagents

1. Amino acids standard solution

0.1 Molar solution of each amino acids was made by dissolving 1/10th quantity of gram-molecular weight of the amino acid in water to obtain one litre of solution. This was further diluted with water to obtain 10^{-2}, 10^{-3} and 10^{-4} molar solution of the amino acids.

2. Ninhydrin (locating reagent)

A 0.2 % ninhydrine was prepared by dissolving 0.2g ninhydrin in a few ml of acetone (A.R.) and volume made up to 100 ml with acetone.

3. Orthophthalaldehyde (OPA) -2-mercaptoethanol (2.ME) (Locating reagent I)

A 0.1M solution of *o*-phthalaldehyde (OPA) was prepared by dissolving accurately measured

1.34g of it in 100 ml of acetone. This was then diluted with acetone to obtained 10^{-2}, 10^{-3}M solution of OPA. A freshly prepared 0.1% v/v of 2-merceptoethanol (2-ME) in water was added to it (0.2 ml per 10 ml of 10^{-2}M OPA solution) before spraying.

4. Buffer solution, pH 10.0

A 4.80 g of borax and 0.80 g of sodium hydroxide were dissolved in distilled water and diluted to 900 ml with distilled water. The pH was checked with a pH meter and adjusted to 10.0 if necessary, by using either 1M sodium hydroxide or 1M hydrochloride acid. The final volume of the buffer was made to one litre with distilled water.

C Sample preparation

Fresh oranges, lemons, tomatoes and apples were washed with distilled water, cut into two halves and pressed to extract juice. A 10 ml of the juice from any of the fruit was treated with 3 ml of ethanol (to deproteinase, to desalt) and allowed to stand for 10-15 minutes; centrifuged off any precipitate and filtered. The filtrate was collected in separate reagent bottles, covered with glass stoppers and stored.

D. Solvent systems for developing chromatogram

The following solvent mixtures were used as mobile phase: Solvent I: *n*-butanol: acetic acid : water 12:3:5. Solvent No. II Phenol : water (500 g of phenol to which 125 ml of water and a trace of ammonia were added). Solvent No. III: *n*-butnaol:acetic acid: water 5:2:3

E. Separation of amino acid and their quantitation

(a) Method

Chromatographic paper (Whatman No. 1) of size 35 x 5 cm or 20 x 20 cm was used. At one edge of the paper approximately 1.5 cm above from the lower edge it was marked with pencil and also marked out the position for the application of sample. Capillary tube was used for applying small drop of each standard solution as well as the real sample solution at the specified positions. Warm air draught from a hair-dryer was used to dry the solution spots. The lower edge of the chromatographic paper was immersed into a suitable solvent contained in a vessel previously saturated with vapours of the mobile phase. It was, however, assured that the solute spots did not touch the solvent system and the chromatographic jar was covered with a lid and allowed to stand for 2-3 hours for developing. After developing the paper was dried in air and then with hot air draught from a hair-dryer. For analysis of fruit juices the prepared sample was used and the above procedure was followed (see sample preparation).

(b) Identification

The dried chromatographic sheet was then sprayed with the locating reagent (ninhydrin or OPA/2ME). The paper was then dried with dryer and then heated in an oven at 110°C for five minutes. An outline around the dried coloured spots was drawn by pencil. R_f (retardation factor) values were then determined for each of the spots to identify the amino acids by comparing with the standards.

(c) Measurement

(i) Absorptiometry

Equal areas of the sprayed paper both for the sample and the blank (without sample) were cut and placed separately into cold water (10 ml). These were shaken vigorously and the clear solutions decanted. The absorbance of the sample solution was measured against the blank at the wavelength of maximum absorbance 340 and 350 nm in the case of ninhydrin and OPA-amino acid derivatives respectively.

The quantity of amino acid in a sample was obtained using the previously prepared standard calibration curve.

(ii) Measurement of spot area

a. The zone are of the developed spots was transferred to a piece of graph paper and the squares were counted.

b. The zone area of the spots was obtained by evaluating integral $\int_0^4 x(4-x)dx$ of the shaded portion (Fig. 3(vi)).

c. The size of the spots is also estimated by copying the spots on a sheet of good paper, cutting out, and weighing.

(d) Removal of spots from the chromatogram

The appropriate area of the chromatogram containing solute was cut out and treated with the proper solvent e.g. water and volume made upto the mark (25 ml) in a suitable measuring flask using the same solvent. The eluted component was then determined by absorptiometric method.

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