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Short Communication

Thin-layer chromatographic method for the simultaneous determination of physiological aromatic amino acids

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ABSTRACT

A method for the determination of phenylalanine, tryptophan and tyrosine has been adapted from a published method based on thin-layer chromatographic separation on cellulose. The proposed method uses sodium sulphate solution as the migration solvent, which avoids the complete plate coloration that occurs with ninhydrin and leaves only stained spots where amino acids are present. Quantification is performed by densitometry. A linear relationship between amino acid concentration and peak area is obtained for amounts in the range 0.1–1 μg of each amino acid. The method is rapid (25 samples within 6 h), it is specific for aromatic amino acids and it is economical. The main disadvantages are that three standards per plate are needed and that the geometry of the spots is influenced by the ionic strength of the solution, necessitating the preparation of the further standard amino acid solution at a similar ionic strength.

INTRODUCTION

The identification and determination of amino acids and protein sequencing have remained important aspects of biology [1] This group of molecules is also of great importance in clinical biology and pharmacology [2]. Within the nutrition field, essential amino acids play an important role [3] and are considered as industrially interesting metabolites [4]. They include tryptophan, which interposes in brain functioning [5] and which is expensive [4]. Phenylalanine and tyrosine are also of importance and are related. In fact, their biosynthesis is cross-regulated in both prokaryotic

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and eukaryotic cells [6]. This makes the simultaneous determination of these three aromatic amino acids of considerable interest.

The aim of this work was to improve the sensitivity of the thin-layer chromatographic (TLC) method published by Kuhn and Lederer [7] and to adapt it to densitometric determination.

EXPERIMENTAL

Materials

Glass TLC plates (20 × 20 cm) with a cellulose layer 0.1 mm thick were obtained from Merck (Darmstadt, Germany) and tryptophan, tyronsine, phenylalanine and ninhydrin from Fluka (Buchs, Switzerland). The densitometer used was a Shimadzu CS 9000 from Roucaire (Vélizy-Villacoublay, France).

Procedures

A standard solution was prepared dissolving 100 mg of each amino acid in 1 l of distilled water. Volumes of 1–10 μl of the standard or the sample solution were placed with a 20-μl micropipette 2 cm from the edge of the plate every 1.5 cm. Each deposit was performed using small amounts, separated by drying periods. The plates were then placed in a developing tank that had been saturated 1 day before with sodium sulphate solution in distilled water. The ascending mode was used and the migration was stopped when the solvent line reached 12 cm from the sample deposit line. The migration time was about 60–90 min. After drying in an oven at 110°C for 10 min, the spots were revealed by spraying with a 0.3 g in 100 ml solution of ninhydrin in acetone in such a way that the plate was completely wetted. The volume sprayed was about 20 ml. The plates were then incubated at 110°C for 15 min. When the plate had cooled to room temperature, the absorbance of the spots was immediately measured using the densitometric analyser under the following conditions: photo mode, absorbance reflection; scan mode, zig-zag; wavelength, 565 nm; zero set mode; background correction; swing width, 12.0 mm; δy, 0.04 (δy is a parameter of the swing, as noted by the constructor); peak filter, 2; drift line, 0; minimum width, 5.0; minimum area, 5000.0.

RESULTS

Optimization of migration solvent

Because of the reaction of the ammonium sulphate used in the original procedure [7] with ninhydrin, we proposed to replace it with sodium sulphate solution. However, a 1.3 *M* concentration did not give a good separation. Table I shows the R_F values of tryptophan, tyrosine and phenylalanine on plates where the migration step was achieved with sodium sulphate solutions of different concentrations. The optimum value is assumed to give the best separation and to give R_F values below 0.8, in order to avoid interactions between the spots and the solvent front. The optimum sodium sulphate concentration was 0.7 *M*. Fig. 1 shows a plate on which tryptohan, tyronsine and phenylalanine were separated with 0.7 *M* sodium sulphate solution and developed with ninhydrin.

TABLE I

OPTIMIZATION OF THE SODIUM SULPHATE CONCENTRATION OF THE MIGRATION SOLVENT

Other conditions as indicated under Experimental.

Sodium sulphate concentration (M)	R_F values		
	Trp	Tyr	Phe
0	0.5	0.77	0.86
0.5	0.47	0.76	0.83
0.7	0.43	0.71	0.78
0.9	0.38	0.67	0.74
1.3	0.33	0.64	0.68

Densitometric analysis

Fig. 2 shows a chromatogram given by the densitometer under the optimum conditions.

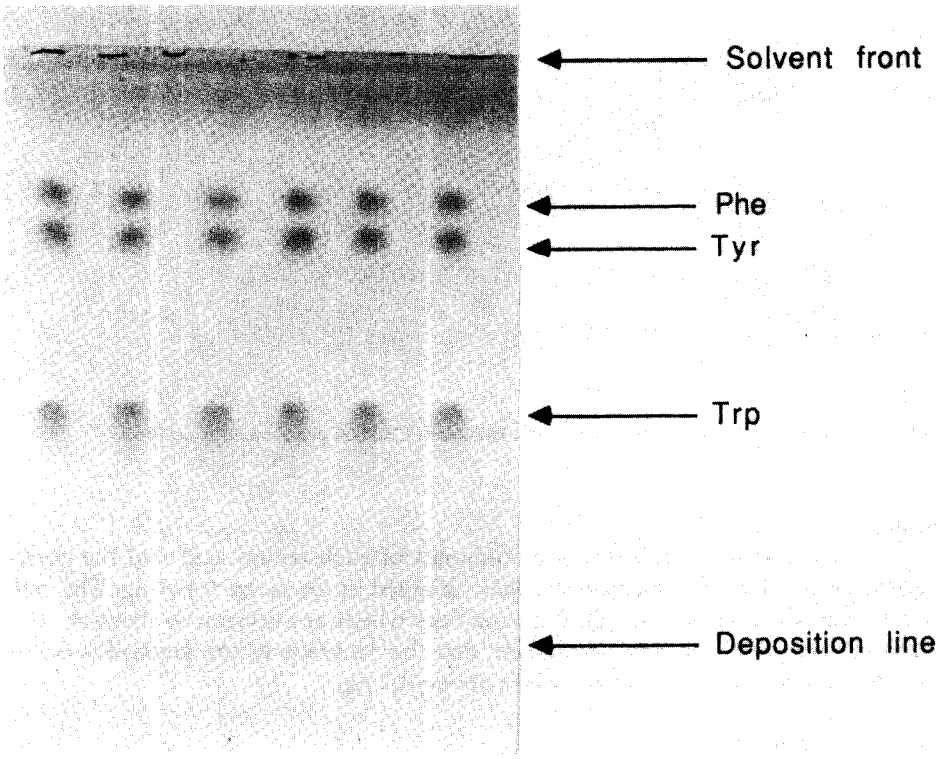

Fig. 1. Separation of phenylalanine, tyrosine and tryptophan in a mixture containing 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 μg of each amino acid on a cellulose thin-layer plate. Assays were performed as indicated under Experimental.

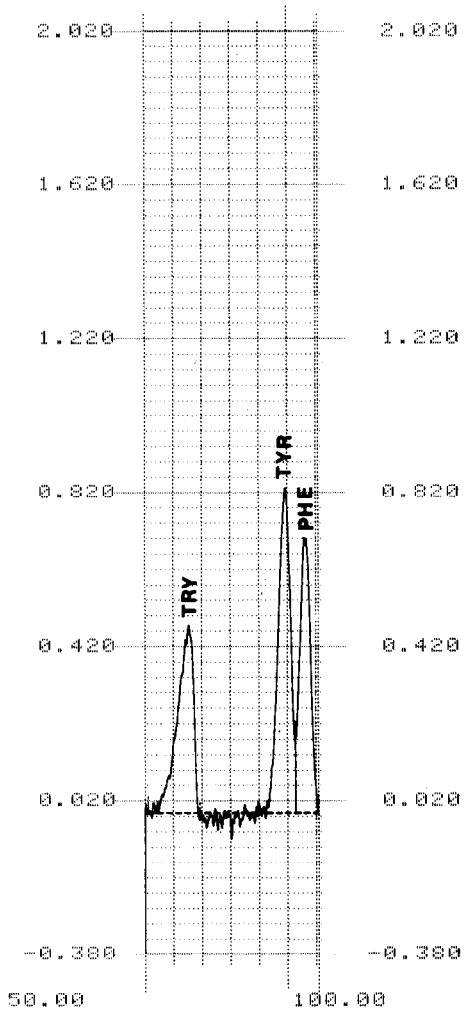

Fig. 2. Example of a chromatogram given by the Shimadzu CS 9000 densitometric analyser.

Linearity

A linear relationship between the amount of each amino acid and the corresponding peak integrated surface area was obtained in the range 0.1–1 μg. The calibration graphs are shown in Fig. 3. Error bars which are arbitrarily fixed at 10% around the integrated area value indicate that the accuracy of the method is below 10% when the amount of amino acids is above 0.2 μg.

Sensitivity and accuracy

The method is able to detect amino acids in amounts down to *ca.* 0.1 μg. As the maximum recommended volume is 10 μl, tryptophan, tyrosine, and phenylalanine can be determined in solutions in concentration down to 10 mg/l.

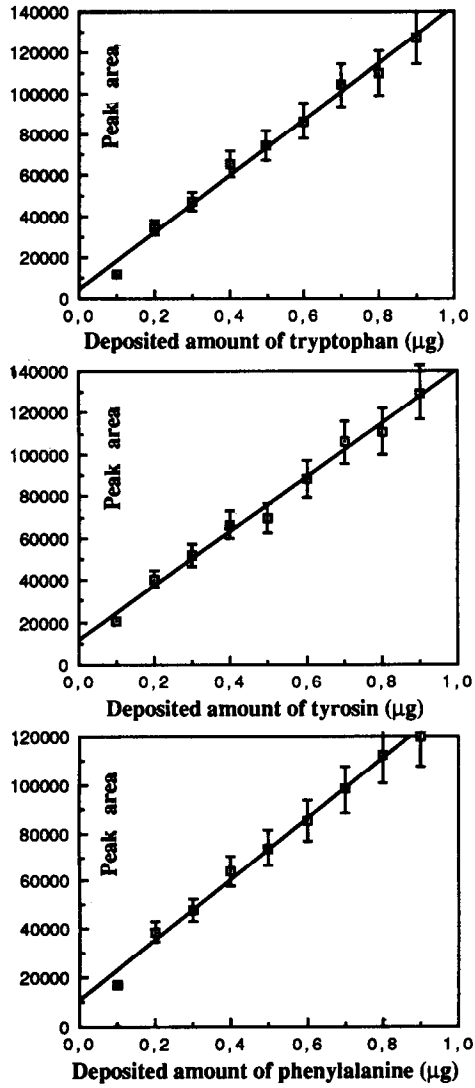

Fig. 3. Calibration graphs obtained for each amino acid. Assays were performed as indicated under Experimental. Error bars around area values are arbitrarily fixed at 10%.

Solutions containing 0.1, 0.4, 0.6 and 1 μg of a mixture of the three aromatic amino acids were spotted four times on a plate and analysed. The average, standard deviation and relative standard deviation of the integrated areas were calculated and the results are given in Table II.

The standard deviation, which is the absolute error, does not depend on the amount of amino acid applied.Thus the relative standard deviation, which is the relative error, increases strongly when the amount decreases. The relative error is below 10% when the amount deposited is up to 0.1 μg, except for tryptophan, because the geometry of the spots increases the integration errors.

TABLE II

STATISTICAL ANALYSIS OF THE RESULTS FOR DIFFERENT AMOUNTS OF EACH AMINO ACID APLLIED

The calculations were made with four deposits for each amount. Assays were performed as described under Experimental.

Amino acid	Statistical parameters[a]	Amount applied (μg)			
		0.1	0.4	0.6	1.0
Tryptophan	μ	18057	55223	73088	132670
	Σ	2547	4304	3669	1175
	Σ/μ (%)	14.1	7.6	5.0	0.9
Tyrosine	μ	26143	65893	86601	131035
	Σ	2939	2393	3834	3460
	Σ/μ (%)	11.2	3.6	4.4	2.6
Phenylalanine	μ	18386	48800	69837	118298
	Σ	1744	1673	1459	1234
	Σ/μ (%)	9.4	3.4	2.1	1.0

[a] μ = Average of the integrated area; Σ = standard deviation; Σ/μ = relative standard deviation.

DISCUSSION

Advantages

In addition of the accuracy and sensitivity which were indicated above, the technique has the following advantages: no treatments are necessary before the chromatographic analysis; only aromatic amino acids are separated because the others remain in the solvent front; the materials that are needed are inexpensive so that this method can be used in routine applications; and is rapid, since two plates (24 samples) can be treated in 6 h. In addition, the densitometer has an automatic analysis function.

Selectivity

The support–solvent system used and the ninhydrin reaction decrease interference problems. However, some molecules from biogenic amines such as dopamine or tyramine have R_F values between those of tyrosine and phenylalanine [2] and could interact in a quantitative determination.

Limitations

Ninhydrin development can differ from one plate to another. Therefore, it is necessary to include three standard solutions at different concentration on each plate, which decreases the number of samples that can be treated per plate. Moreover, ionic strenght can influence the geometry of the spots, particularly for phenylalanine and tyrosine. This requires standard solutions to be prepared at the same ionic strength as the samples.

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