SEPARATION OF 3-METHYLHISTIDINE FROM HISTIDINE BY THIN-LAYER CHROMATOGRAPHY

A RAPID METHOD FOR DETECTING WHALE EXTRACT IN SOUP PRODUCTS

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During our studies on soup products, it was necessary to devise a method for the detection of whale meat extract in these products.

On assessing the β-alanylhistidine dipeptides by published methods[1,2] a marked difference was observed between whale and beef extracts in that whale extract had a much higher total dipeptide content than beef extract.

We have recently received information that POCCHIARI *et al.*[3] have found a substantial difference not only quantitative, but also qualitative, between the dipeptides of whale and beef extracts. While beef extract shows a predominance of carnosine (β-alanylhistidine) and a small amount of anserine, (β-alanyl-1-methylhistidine) whale extract contains not only carnosine, but also a considerable proportion of β-alanyl-3-methylhistidine.

It was therefore necessary to devise a system capable of separating 3-methylhistidine from histidine and a rapid method based on thin-layer chromatography has been adopted.

EXPERIMENTAL

Chromatoplates and solvents

The chromatoplates were prepared according to the method described by LEES AND DE MURIA[4]. The one-dimensional plates (24 × 8.5 cm) were covered with an 0.2 mm layer of Silicagel G (E. Merck, A. G., Darmstadt) and the two-dimensional (15 × 15 cm) with an 0.15 mm layer. They were dried in air for 2 h, without activation in the oven.

Solvent No. 1, methanol–pyridine–water–glacial acetic acid (6:6:4:1 v/v), was used for the one-dimensional run.

Solvent No. 2, phenol–ethanol–water–ammonia (3:1:1:0.1 v/v), was used for the first direction in the two-dimensional run, while solvent No. 1 was used for the second direction.

Developers

A: Polychromatic developer proposed by MOFFAT AND LYTLE[5].

B: Solution containing 0.2 g of ninhydrin and 0.02 g of hydrindantin in absolute ethyl alcohol–glacial acetic acid (80:20 v/v).

0.5 ml of pyridine was added to 100 ml of the reagent prepared as above.

The hydrindantin was prepared according to the method of MOORE AND STEIN[6].

Preparation of 3-methylhistidine

Pure 3-methylhistidine was prepared as follows. Histidine was converted to phthaloyl-histidine by the method of SHEEHAN AND FRANK[7], as these authors applied it in the preparation of phthaloyl-DL-phenylalanine. The phthaloyl-histidine was methylated as described by TALLAN *et al.*[8].

3-Methylhistidine was separated from the solution containing histidine, 1-methyl-histidine and 3-methylhistidine on a column of Amberlite resin IR 120 C.G. fraction C

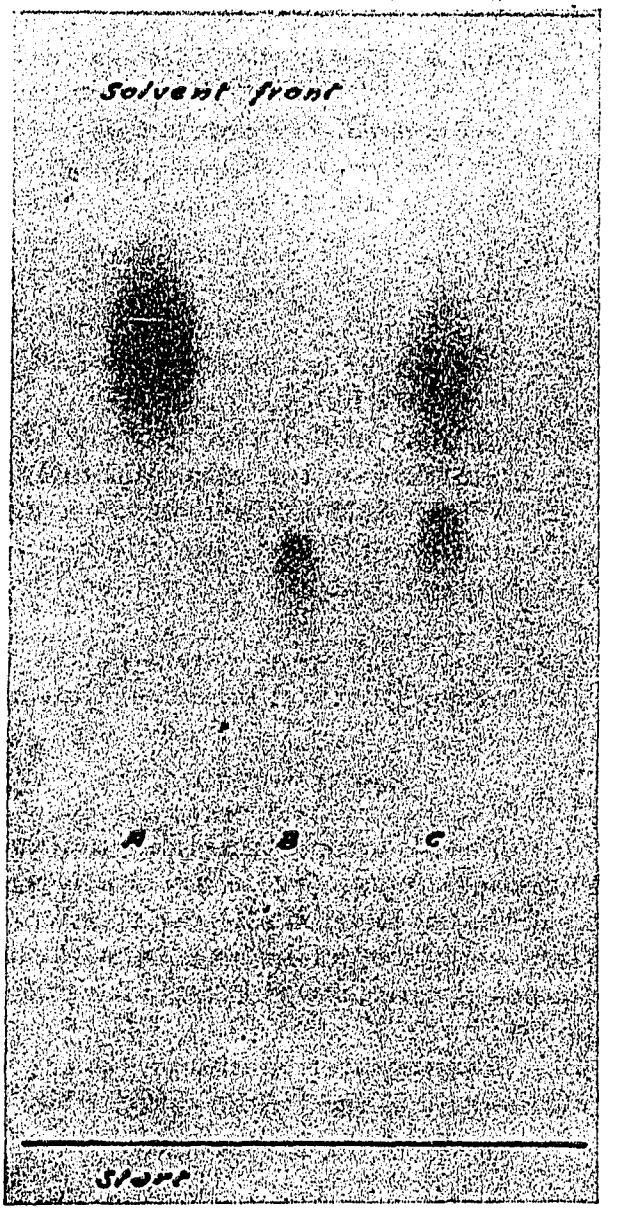

Fig. 1. Thin-layer chromatography on Silicagel "G". 15 cm run; solvent No. 1. A = tablet containing 15% beef extract; B = 3-methylhistidine; C = tablet containing 12% whale extract.

(50 cm high, 0.9 cm diam.) by the method of MOORE *et al.*[9] for the separation of basic amino acids. The combined fractions containing 3-methylhistidine were desalted on a column (14 cm high, 0.9 cm diam.) of Amberlite resin IR 120 C.G. fraction E (H form).

The resin was washed with 80 ml of distilled water and the 3-methylhistidine was eluted with 50 ml of 1.5 *N* NH_4OH. The flow rate was 60 ml/h.

Preparation of the solutions under examination and chromatographic procedure

The amount of total dipeptide contained in the product under test was determined by the proposed method[2]. A quantity equivalent to 0.075 g of dipeptides was dissolved in

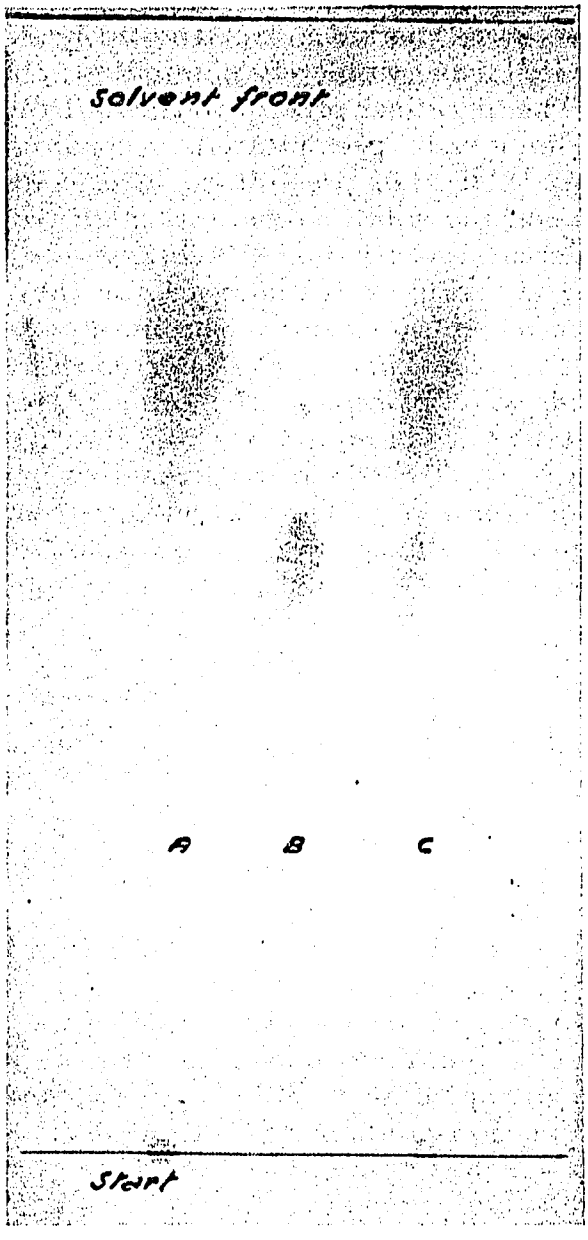

Fig. 2. Thin-layer chromatography on Silicagel "G", 15 cm run; solvent No. 1. A = tablet containing 15% beef extract; B = 3-methylhistidine; C = tablet A + 5% whale extract.

25–30 ml of hot water and then left to cool under running water until the fat had solidified. The liquid was filtered through glass wool into a 100 ml flask containing 5 g of trichloracetic acid dissolved in a little water. The solution was diluted to 100 ml

and, after shaking, allowed to stand for 15 min. All the solution was transferred to an Erlenmeyer flask with a ground glass neck containing 2 g of kieselguhr, shaken for 2 min, and then filtered through a Whatman No. 40 filter paper.

5 ml of the resulting filtered solution was transferred to a 20 ml vial with thick walls, and 5 ml of concentrated HCl was added. The whole was heated on a waterbath for 5 min after which the vial was sealed in a flame. The vial was then placed in a 250 ml flask having a ground glass neck and containing 150–200 ml of methylcellosolve (ethylene glycol monomethyl ether) and refluxed for 2 h. The flask was cooled and the vial opened. The contents were poured into a 100 ml beaker and dried on a boiling water bath until all the HCl was completely eliminated; the residue was taken up in 10 ml of distilled water. The histidines present in the hydrolysate (histidine, 3-methylhistidine, 1-methylhistidine) were precipitated, according to the β-alanylhistidine dipeptide precipitation method[2]. The precipitate, after centrifuging, was dissolved in concentrated HCl. The resulting solution was saturated with H_2S, filtered through a Whatman No. 41 filter paper, and dried on a boiling water bath until all the HCl was completely eliminated. The residue was taken up in 3 ml of distilled water and 3 μl of the solution placed on a one-dimensional chromatoplate at a distance of 1.5 cm from one of the shorter ends. A few μl of a solution containing a suitable concentration of pure 3-methylhistidine were placed on the same plate. The chromatoplate was placed vertically in a suitably sized museum jar containing solvent No. 1. The solvent front was allowed to reach exactly 15 cm from the starting point and the plate was placed in an oven with forced air circulation, at 85° for 30 min. The plate was allowed to cool, the developer was carefully sprayed on to the surface, and the plate was then returned to the oven for the time required by the developer employed (see Table I).

TABLE I

TIMES AND TEMPERATURES USED FOR DETECTION WITH DEVELOPERS A AND B

Developer	*Time (min)*	*Temperature °C*
A	10	110
B	5	85

For two-dimensional chromatography 9 μl of the test solution was placed in the lower left-hand corner of the plate (15 × 15 cm) at 1.5 cm from the two edges. Two lines, crossing at right angles, are drawn in pencil at a distance of 11.5 cm from the lower edge and from the left edge. These lines, which cross the whole adsorbent surface from left to right and from top to bottom respectively, represent the limit of run of the solvents in the two directions and form two thin adsorbent channels. Two spots of standard 3-methylhistidine solution are placed on these channels, at the same height as the unknown sample. Each of the spots, running in a single solvent, will indicate the position reached by 3-methylhistidine in that solvent (see Fig. 4). The chromatoplate was placed vertically in a suitably sized museum jar containing solvent No. 2 for the first run. When the solvent front reached the limiting line, the plate was placed in an oven with forced air circulation at 55° for 45 min. After cooling, the plate, rotated through 90° was placed in another similar museum jar containing solvent No. 1 for the second run. When this solvent also had reached the limiting line, the plate was re-

moved and dried in an oven at 85° for 30 min. After cooling, the whole silica gel surface was sprayed with the developer and then returned to the oven for the time required by the developer used.

RESULTS AND DISCUSSION

During the present work we observed that the histidine spot in one-dimensional chromatography is often accompanied by other spots having only a slightly different R_F, but it is easily recognisable by the violet colour at the edges and the reddish centre with a yellowish halo when the chromatogram is developed with freshly prepared developer A.

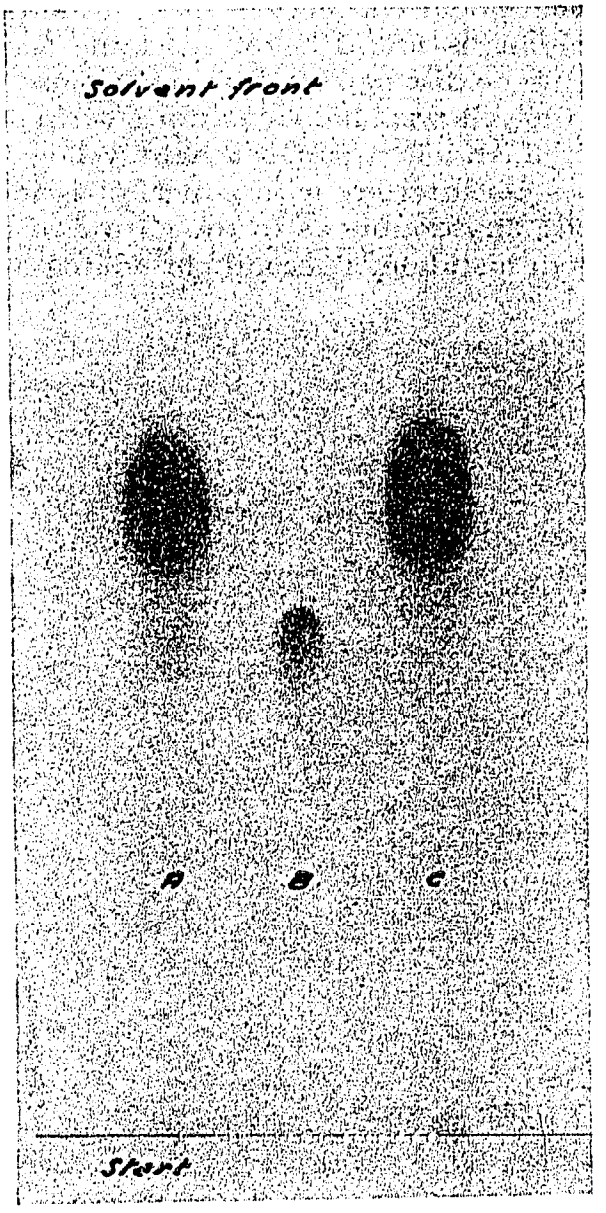

Fig. 3. Thin-layer chromatography on Silicagel "G". 15 cm run; solvent No. 1. A = tablet containing 14% beef extract + 1% whale extract; B = 3-methylhistidine; C = tablet containing 15% beef extract.

TABLE II

R_{His} VALUES OF HISTIDINE, 3-METHYLHISTIDINE AND 1-METHYLHISTIDINE

Amino acid	$R_{His} \times 100$	
	in solvent No. 2	*in solvent No. 1*
Histidine	100	100
3-Methylhistidine	153	84
1-Methylhistidine	156	86

The values represent the average of 6 runs.

Still referring to one-dimensional chromatography, 3-methylhistidine together with 1-methylhistidine, if present, is found below histidine (see Table II). However, 3-methylhistidine can readily be distinguished by the intense blue-violet colour, almost bordering on black, and the yellowish halo it assumes when developer A is used. Under these conditions, 1-methylhistidine assumes a light yellow colour, sometimes tinged with green, and thus does not interfere with 3-methylhistidine.

If, however, there are any doubts as to the nature of the methylhistidine separated by this method from histidine, a second chromatogram can be developed with

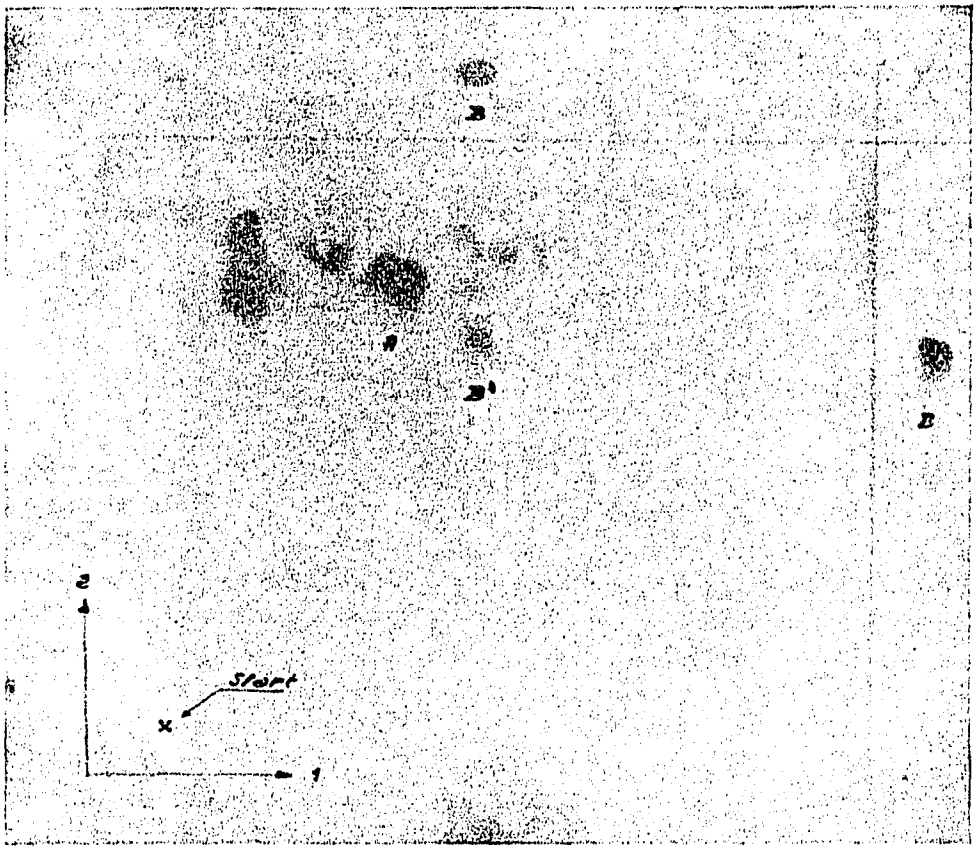

Fig. 4. Two-dimensional thin-layer chromatography on Silicagel "G". 10 cm run; 1st direction solvent No. 2, 2nd direction solvent No. 1. Tablet containing 14% beef extract + 1% whale extract with 3-methylhistidine control. Loading 9 μl. A = histidine; B = 3-methylhistidine (control); B^1 = 3-methylhistidine from whale meat extract.

developer B. In this case, 3-methylhistidine gives a red-brown spot, and 1-methylhistidine a bluish-grey colour tinged with violet.

Relatively large amounts of histidine interfere with the detection of small amounts of 3-methylhistidine by one-dimensional chromatography because of the tail produced by the former when run in solvent No. 1 (see Fig. 3). In such cases, two-dimensional chromatography should be used to separate the 3-methylhistidine from the histidine tail (see Fig. 4). By this means we can detect 1 μg of 3-methylhistidine associated with 15 μg of histidine. Comparison of R_F values and colour reaction with the pure compound will assist identification.

SUMMARY

A rapid method for separating 3-methylhistidine (and possibly 1-methylhistidine) from histidine by means of thin-layer chromatography is described. It can be used to detect 1 μg of 3-methylhistidine associated with 15 μg of histidine. The R_F values relative to histidine (R_{His}), of 3-methylhistidine and 1-methylhistidine in the solvents used, are also given.

The method has been devised in order to detect whale meat extract in soup products, even when it is mixed with beef extract.

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