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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF ATP AND THE PRODUCTS OF ITS DEGRADATION IN MEAT TISSUE

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SUMMARY

A method has been developed for the quantitative analysis of adenosine triphosphate and the products of its degradation in *post mortem* meat tissue, by means of thin-layer chromatography and fluorescent quenching techniques.

INTRODUCTION

In recent years it has become increasingly apparent that the products of ATP* degradation in *post mortem* meat tissue merit closer investigation by the food scientist since it has been suggested that these breakdown products contribute to the flavour (Doty *et al.*[1], Jones[2], Disney *et al.*[3]) eating quality (Howard *et al.*[4]), and water-holding properties (Hamm[5,6] and Lawrie[7]) of meat.

Until recently, it has been difficult to quantify ATP and all its degradation products to Hypox in meat tissue within a single analysis. Hurlbert *et al.*[8] developed a method by which nucleotides could be separated and quantitatively determined using ion-exchange techniques. Jones[9] subsequently modified and extended this procedure for the analysis of nucleotides, nucleosides, and purines in fish muscle, but the method was time-consuming and required two separate ion-exchange procedures. Bendall and Davey[10] also devised a method based on ion exchange but this suffered from the same faults and was rather limited in application.

Enzymatic analysis has also been used to measure ATP and some of the isolated intermediates derived from it (Bergmeyer[11]), but these methods are not favoured because the experimental procedures involved are often complicated and because the cost of dealing with large numbers of samples is high.

Recently Potthast and Hamm[12] developed a new procedure based on thin-layer chromatography (TLC) in which ATP and its intermediates were separated on silica gel coated plates impregnated with a fluorescent material. This method has the advantage that all of the intermediates from one meat extract can be separated and quantitatively measured on one TLC plate during a single analysis.

Initial trials carried out by the present authors with the TLC method showed that although good separations were achieved with standard solutions, practical difficulties arose when meat extracts were assayed. Investigation has since shown,

* The following abbreviations have been used throughout this paper: ATP, ADP, AMP = adenosine tri-, di- and monophosphate; IMP = inosine monophosphate; Ino = inosine; Hypox = hypoxanthine; PCA = perchloric acid.

however, that difficulties and disadvantages in the procedure of Potthast and Hamm can be obviated by introducing the appropriate modifications detailed and discussed in this paper. Incorporating these modifications has produced a fast and accurate method for separating and quantitatively measuring ATP and the products of its degradation in meat tissue.

EXPERIMENTAL

Materials

All biochemical substrates were obtained from Boehringer (London, Great Britain). TLC plates were 20 × 10 cm silica gel F_{254} type, supplied by Anderman (London, Great Britain).

Organic solvents were purchased from either E. Merck (Darmstadt, G.F.R.) or British Drug Houses (Poole, Dorset, Great Britain).

The instrument used for spectrofluorometric measurements was the Vitatron TLD 100 universal densitometer supplied by Fisons (Loughborough, Great Britain). All other chemicals were purchased from British Drug Houses and were of AnalaR grade.

Constriction pipettes (25 μl) and chromatographic tanks (22 cm × 7 cm × 22 cm depth) were purchased from Shandon (London, Great Britain).

Extraction of meat tissue

The procedure for obtaining a deproteinised meat extract relies upon the action of 5% (v/v) PCA to denature any protein material and at the same time to extract all of the soluble components of the meat tissue[13]. The meat sample is stored and later powdered in liquid nitrogen, and approximately 1 g macerated in 8 ml of ice-cold 5% PCA. A further 8 ml of PCA are added to the macerate and the whole is allowed to stand for 20 min before it is filtered through a Whatman No. 42 filter paper. 10 ml of the supernatant is adjusted to pH 3.0–4.0 with 5 *M* potassium carbonate, using thymol blue as indicator. (At the correct pH the indicator colour change is from red to yellow.)

It is essential to bring the extracts to this pH since when the chromatogram is developed it has been observed that (i) in unneutralised extracts the purine derivatives move rapidly with the leading solvent front with little or no separation and (ii) with a pH greater than 7.0 the derivatives remain aggregated just above the base line.

'Neutralised' extracts are stored at 0° for at least 30 min before use, to allow the precipitate of potassium chlorate to settle out. The clear supernatant thus obtained is used directly for the TLC separation.

Stock standard solutions

Stock standard solutions (10 μmoles/16 ml; this adequately covers the observed range of concentrations of each compound present in 1 g of meat tissue) are prepared by dissolving the individual compounds in glass-distilled water and adjusting the pH to around 4.0 (PCA or K_2CO_3 as appropriate) before making up to 100 ml.

Allowance should be made for the percentage purity and water of hydration of the commercially prepared substrates.

All compounds readily dissolve in distilled water, with the exception of Hypox, which requires alkaline conditions (K_2CO_3) for solvation, followed by acidification to pH 4.0 with PCA prior to making up to the final volume. These stock solutions are diluted to give a further series of standard solutions with concentrations equivalent to 8, 6, 4, 2, and 1 μmoles/16 ml.

Spot application

Extracts and standard solutions are applied to the chromatographic plates using 25-μl constriction pipettes. It is important that the spot diameter is kept to between 8–9 mm by applying the 25 μl as ten individual drops, ensuring that each spot is dry before a further application is made to the plate.

Formulation of solvent system

Although standard solutions of ATP and its intermediates gave adequate separations with the multi-component solvent system of Potthast and Hamm[12], poorer separations were obtained with meat extracts. It was therefore necessary to modify the solvent system. A most satisfactory separation was obtained with the system isobutyl alcohol–amyl alcohol–ethoxyethanol–ammonia–glass-distilled water (15:10:30:15:25).

Plate development

Plates are developed in a tank containing 100 ml of the solvent system. Well defined separations are achieved if: (1) The solvent system is 'matured' for at least 48 h before use; (2) The solvent system is added to the tank 1 h before introduction of the plates; (3) The tank is placed in a constant environment to minimise the effects of draughts, sunlight and uneven heating; (4) When more than one plate is being developed, the silica gel layers are placed facing one another. This eliminates the increased rise of the solvent at the plate edges resulting in a concave-shaped solvent front at the end of the run.

Principle of measurement

Quantitative measurement of the separated compounds relies upon fluorescent quenching, which measures the loss in intensity of fluorescent light emitted from an activated surface by light of a shorter wavelength. Fluorescence in this instance is produced by fluorescein-impregnated silica gel which emits green light ($\lambda_{max.}$ = 526 nm) when exposed to ultraviolet (UV) light at a wavelength of 254 nm.

ATP and its intermediates present in the silica gel layer appear blue due to the absorption characteristics of these purine-based compounds in UV light[14]. The degree to which the emitted green light is masked by the blue is a function of the concentration of the compound on the plate.

Instrumentation

The Vitatron TLD 100 is specifically designed for the quantitative measurement of compounds separated as spots on TLC plates and where the distribution of the compound is not necessarily homogeneous within the spot.

The separated spot, which may not be homogeneous, and which need not have a particular shape either, can be considered to be composed of many smaller spots which may be regarded as homogeneous. The quantity of light reflected from each of the smaller spots is measured individually, all values are integrated, and the averaged value passed continuously to the recorder.

As the sample on the plate is scanned horizontally by the incident light beam, a curve is produced on the recorder chart the area of which may be related directly with the amount of material on the plate. The recorder is fitted with an integrator and the area is determined as the number of integrator units under the curve.

Spot alignment and spot density measurement

The TLC plate is secured such that the incident light beam passes through the centre of each of the separated compounds. Initial marking of the TLC plate at the top and the bottom of each separation run facilitates this alignment.

The light beam of the densitometer is positioned onto a blank area of the plate which has been developed by solvent. The light shutter is then moved into a position which blocks the transmitted light from the plate to the measuring unit, and the recorder is set to full-scale deflection (0% transmission). The light shutter is now opened and the recorder pen set to give zero deflection, *i.e.* 100% transmission. Each plate is calibrated in this way prior to quantitative measurement of the spots.

The loss of green fluorescent light, due to the presence of the blue spots, is recorded as a series of peaks, the areas of which are measured in arbitrary integrator units.

RESULTS

Separation

The following $R_F \times 100$ values were obtained for ATP and each intermediate: ATP, 26; ADP, 35; AMP, 44; IMP, 52; Ino, 65; Hypox, 72.

Calibration curves

Table I shows the mean areas and standard deviations (S.D.), expressed in integrator units, recorded for standard solutions of ATP and its degradation products. Coefficients of variation are also shown in the table.

It can be seen from Table I that the mean values for ATP and AMP were similar as were those for IMP, Ino, and Hypox. Accordingly, composite curves were produced and these are shown in Fig. 1, together with the curve for ADP.

Recovery trials

Volumes of the stock ATP solution were added to neutralised 5% perchloric acid extracts of beef muscle to produce a series of solutions with added ATP concentrations equivalent to 10.0, 5.0, 2.5, 1.3, and 0.6 μmoles/16 ml (or 1 g of meat). An extract blank was also included in the series. 25-μl aliquots of each solution were applied to the TLC plates as six individual spots and these were developed and quantitatively measured. ATP concentrations were calculated using the composite calibration curve for ATP/AMP. Mean recoveries of added ATP, standard deviations and coefficients of variation for the different concentrations are shown in Table II.

TABLE I

PEAK AREAS RECORDED FOR STANDARD SOLUTIONS OF ATP AND RELATED COMPOUNDS

Intermediate	*Number of plates measured*		*Concentration (μmoles/16 ml)*					
			1	*2*	*4*	*6*	*8*	*10*
ATP	10	Mean	21.3	40.3	59.4	76.6	95.8	107.9
		S.D.	2.9	3.9	2.2	3.9	1.5	4.8
		Coefficient of variation, %	14	10	4	5	2	4
ADP	13	Mean	17.4	27.5	40.1	51.2	59.9	67.3
		S.D.	1.7	1.7	3.0	5.0	4.5	4.7
		Coefficient of variation, %	10	6	7	10	7	7
AMP	10	Mean	20.2	38.2	62.0	81.4	94.0	104.8
		S.D.	3.4	3.1	1.8	2.1	4.9	3.8
		Coefficient of variation, %	17	8	3	3	5	4
IMP	12	Mean	10.5	21.1	38.9	56.4	68.0	78.3
		S.D.	2.0	1.9	2.4	3.4	5.2	5.0
		Coefficient of variation, %	18	9	6	6	8	6
Ino	10	Mean	12.8	23.0	40.5	54.2	64.4	74.4
		S.D.	1.4	2.6	3.4	4.7	6.9	6.4
		Coefficient of variation, %	12	12	8	9	11	9
Hypox	8	Mean	10.5	21.8	39.3	51.6	63.5	72.1
		S.D.	1.6	1.4	2.5	1.8	2.5	1.4
		Coefficient of variation, %	15	6	6	3	4	2

TABLE II

RECOVERIES OF STANDARD SOLUTIONS OF ATP ADDED TO MEAT EXTRACTS

Concentration of added ATP (μmoles/16 ml)	*N*	*Observed recovery (μmoles/ml)*		
		Mean	*S.D.*	*Coefficient of variation (%)*
10.0	6	9.9	0.4	4
5.0	4	4.8	0.2	5
2.5	6	2.6	0.3	11
1.3	6	1.5	0.2	16
0.6	6	0.8	0.1	16

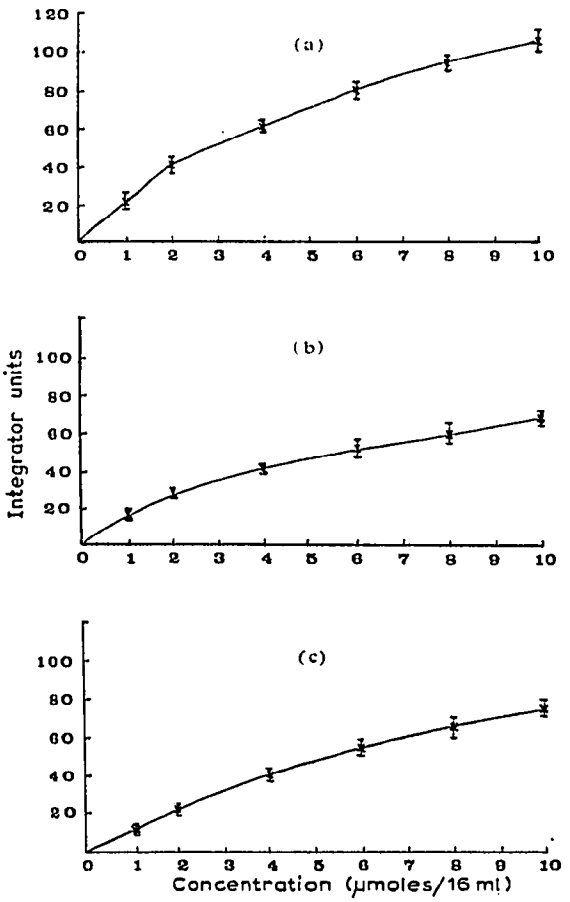

Fig. 1. Relationship between peak area (expressed in integrator counts) and concentration of (a) ATP and AMP, (b) ADP, and (c) IMP, Ino, and Hypox.

DISCUSSION

The original TLC method[12] for separating ATP and its degradation products was found to be satisfactory when using standard solutions but not sufficiently precise with beef extracts to allow accurate quantitative measurement. The streaking effect often observed between the spots is effectively eliminated by adjusting the pH of the extract prior to spotting onto the TLC plate. Furthermore, a more consistent spacing between spots has been achieved by altering the solvent system. These modifications, coupled with the moving spot method of density measurement, enables ATP and all of its degradation products to be quantitatively determined by a single assay procedure. Also, because of the small spot size after development, as many as nine different extracts can now be separated on a single plate.

It was anticipated that the calibration curves for all the compounds would be similar, or more probably that they would bear a direct relationship to one another in proportion to their individual absorption characteristics at a wavelength of

254 nm (ref. 14). However, it is clear from the values presented in Table I that this was not entirely the case. Values for ATP and AMP were similar and could be easily combined as a single curve (Fig. 1a), but the unexpected deviation of ADP (Fig. 1b) (which has identical absorption characteristics to ATP and AMP in UV light) was not easy to explain. The reduced values may result from a supersaturation effect caused by concentration of the nucleotide within a narrower spot area.

Similarities observed in the calibration curves for IMP, Ino and Hypox can be directly related to their corresponding absorption characteristics in UV light[14].

As reported previously[12], none of the calibration curves were found to be linear throughout the range of concentrations studied. In the composite curve for IMP, Ino and Hypox (Fig. 1c) the relationship of concentration to peak area was curvi-linear, whereas in the ATP/AMP composite and ADP calibration curves, straight-line correlations between peak area and concentration were noted up to 2 μmoles/16 ml and above this concentration a less definable pattern.

Although some of the standard deviations presented on the calibration curves may appear to be at first sight excessively high, it may be noted that deviations from the mean value rarely represent concentrations greater than 0.5 μmoles/16 ml or 1 g of meat.

The mean percentage recovery of ATP added to the meat extracts was 107% (S.D. 2%, coefficient of variation 17%). This inordinate level was possibly due to the high observed recovery at lower concentrations of added ATP.

This method has been successfully applied to the determination of ATP and its degradation products in a number of beef hindquarter muscles and the results of this work will be published elsewhere in the near future.

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