STUDIES ON GRASS LIGNINS

I. SEPARATION AND QUANTITATIVE DETERMINATION OF *p*-HYDROXYBENZALDEHYDE, VANILLIN AND SYRINGALDEHYDE BY THIN-LAYER CHROMATOGRAPHY

J. M. BRAND
Department of Biochemistry, Faculty of Agriculture, University of Natal, Pietermaritzburg (South Africa)
(Received September 2nd, 1965)

INTRODUCTION

In the past much attention has been paid to the development of a rapid quantitative method for the estimation of the three phenolic aldehydes, *p*-hydroxybenzaldehyde (P), vanillin (V) and syringaldehyde (S), obtained by the oxidation of isolated lignins or lignified material. Many methods for the separation and estimation of these and other lignin derivatives, obtained by oxidation procedures, have been reviewed by PEPPER AND SIDDIQUEULLAH[1].

In 1951, STONE AND BLUNDELL[2] reported a rapid micromethod for the quantitative determination of P, V and S formed by the alkaline nitrobenzene oxidation of lignin. These three substances were separated by paper chromatography, eluted, and assayed spectrophotometrically. Recently, PEPPER, MANOLOPOULO AND BURTON[3] have described a gas–liquid chromatographic technique for the separation and quantitative estimation of P, V and S.

The application of thin-layer chromatography (TLC) to the quantitative estimation of P, V and S appears to have been neglected. KRATZL AND PUSCHMANN[4] have described the preparative separation of P, V and S by TLC using silica gel G as adsorbent and di-isoamyl ether (water saturated)–*n*-butanol (3:1) as the developing solvent. Various TLC methods have been described for the detection of flavouring compounds, particularly vanillin, in vanilla extract, but most of these methods are not adapted for routine quantitative work. The numerous solvent systems mentioned by SUNDT AND SACCARDI[5] and by KAHAN AND FITELSON[6] for the separation and detection of vanilla flavouring compounds are only suitable for qualitative work with P, V and S.

The TLC separation of the 2,4-dinitrophenylhydrazones of many aromatic aldehydes, including P, V and S, has been reported by RUFFINI[7]. TOWERS AND MAASS[8] have also described the separation of P, V, S and other phenolic substances in silica gel G. A number of these substances have been extracted as such from the *Lycopodiales* while others have been obtained by the alkaline copper oxidation of the lignified material. This paper describes the quantitative determination of P, V and S after TLC separation on silica gel G by a new solvent system. The rapid quantitative extraction of these three substances from aqueous solutions is also described.

EXPERIMENTAL

Phenolic aldehydes

p-Hydroxybenzaldehyde (Fluka), vanillin (British Drug Houses) and syringaldehyde (Fluka) were purified when necessary by repeated recrystallisation until chromatographically pure. Standard solutions of each aldehyde were prepared in 95 % ethanol in concentrations of 10, 25, 75 and 100 μg per 0.025 ml. These solutions were used for the preparation of standard curves. Further standard solutions were made from a mixture of all three aldehydes, each being present in 10, 25, 50, 75 and 100 μg per 0.025 ml. These solutions were used for the determination of percentage recovery after separating the three substances on chromatoplates.

Treatment of silica gel

It was found that syringaldehyde could not be quantitatively extracted from silica gel G (Merck) by ethanol or ethanol acidified with dilute HCl. As this was apparently due to the presence of iron in the silica gel, the silica gel used in this investigation was treated with ethanol–conc. HCl (9:1) for 30 min, washed with ethanol, and dried at 110°. Syringaldehyde could then be quantitatively extracted by ethanol from silica gel treated in this manner.

Standard curves

In the determination of the standard curves for P, V and S it was attempted to simulate the condition which would be encountered during the TLC separation. Approximately 0.4 g silica gel, 0.025 ml of standard aldehyde solution and 10 ml of 95 % ethanol were added to 15 ml centrifuge tubes. A blank tube contained silica gel and ethanol only. The tubes were shaken thoroughly and centrifuged at 1400 $\times g$ for 10 min. The clear supernatant was decanted into further 15 ml centrifuge tubes, each containing 0.05 ml 1.25 *N* KOH. Any precipitate which formed at this stage was removed by centrifugation.

The absorbance of the supernatant solutions was determined in a spectrophotometer (Zeiss PMQII) at the following wavelengths, which correspond to the wavelength of maximum absorption of the ionized form of the three substances: *p*-hydroxybenzaldehyde, 335 mμ; vanillin, 352 mμ; and syringaldehyde 368 mμ. Glass cuvettes were used and the standard curves were found to obey Beer's Law over the concentration range used.

Preparation and development of chromatoplates

The acid-washed silica gel was spread on glass plates (8 in. $\times$ 2 in.) to give layers of 0.3 to 0.4 mm thick. The plates were activated at 110° for 30 min.

The solvent finally selected for the separation of P, V and S was *n*-hexane–isoamyl alcohol (B.D.H.-purified for mild testing)–acetic acid (100:16:0.25). The positions of P, V and S on the chromatoplates were determined by spraying one of the plates with 2,4-dinitrophenylhydrazine (1.0 g in 300 ml concentrated HCl diluted to 1 l with water).

Recoveries from plates

Activated chromatoplates were spotted 2.0 cm from one end with 0.025 ml of

the standard mixtures to give spots approximately 6–8 mm in diameter. The ethanolic spots were allowed to dry and the plates were then dipped into the developing solvent to a depth of 5–7 mm. After development of 15 cm the plates were air dried and the positions of the aldehydes noted by spraying one plate with 2,4-dinitrophenylhydrazine solution. Corresponding zones of adsorbent were carefully scraped, across the complete width of the plate, from the replicate plates into centrifuge tubes. A zone of adsorbent above the solvent front was scraped into a tube to serve as blank. Ethanol (10 ml) was added to each tube, the tubes were thoroughly shaken and centrifuged at 1400 × *g* for 10 min. The supernatant was decanted into further centrifuge tubes containing 0.05 ml 1.25 *N* KOH and the tubes shaken. Any precipitate which formed at this stage was centrifuged down. Absorbance values of the solutions were recorded and the results are presented in Table I.

TABLE I

PERCENTAGE RECOVERY OF P, V AND S FROM CHROMATOPLATES

Concentration (μg)	*Recovery* (%)*		
	P	*V*	*S*
10	102.0	100.0	101.0
25	100.0	99.6	100.0
50	100.0	99.6	100.0
75	98.7	98.0	97.3
100	98.4	99.0	100.0
Mean recovery	99.84	99.24	99.66
S.E.	± 1.40	± 0.78	± 1.40

* Means of three determinations.

Extraction of P, V and S from aqueous solutions

Accurately measured volumes of 2 % aqueous standard aldehyde solutions were pipetted into 25 ml burettes and about 15 ml of saturated ammonium sulphate added. Chloroform (2 ml) was added, the burettes stoppered and thoroughly shaken. When the two phases had separated the chloroform was run into 5 ml volumetric flasks until the aqueous phase reached the bottom of the tip of the burette. The burettes were stoppered, inverted and an iced cloth clasped around the upper half of the burette. The tap was slowly opened and the solution in the tip of the burette sucked back into the burette. The aqueous solutions were again extracted with 2 ml chloroform. The extracts were combined and made up to 5 ml with chloroform. Aliquots of the extracts (0.05 ml) were added to alkaline ethanol and the absorbance read as previously described. The results are presented in Table II.

DISCUSSION

The conventional method of spectrophotometric analysis employing a blank which does not contain the substance to be assayed, was more practicable than the utilisation of difference spectra as suggested by LEMON[9] for the spectrophotometric

TABLE II

PERCENTAGE RECOVERY OF P, V AND S FROM AQUEOUS SOLUTIONS SATURATED WITH AMMONIUM SULPHATE

Concentration (μg)	*Recovery* (%)*		
	P	*V*	*S*
10	98.00	103.00	105.00
25	97.00	100.80	97.00
50	100.00	100.80	100.00
75	100.30	102.00	100.27
100		102.00	100.00
Mean recovery	98.82	101.72	100.45
S.E.	± 1.59	± 0.93	± 2.40

* Means of four separate extractions.

determination of P, V and S. Standard curves of P, V and S obeyed Beer's Law over the concentration range employed.

The solvent employed by KRATZL AND PUSCHMANN[4] for TLC, namely di-isoamyl ether (water saturated)–*n*-butanol (3:1), was found to cause P and V to run too close together for quantitative work. The approximate R_F values obtained with this solvent mixture were: P, 0.85; V, 0.74; and S, 0.35. Many solvents were found to effect a separation of the three substances but P and V generally ran too close together, while S tended to trail. These problems were overcome by using *n*-hexane–isoamyl alcohol–acetic acid (100:16:0.25) as the developing solvent. All three substances appeared as distinct well resolved spots when sprayed with 2,4-dinitrophenylhydrazine (see Fig. 1).

This solvent, however, exhibited the phenomenon known as demixion, or the formation of more than one liquid front[10]. The most rapidly moving part of the solvent was rich in hexane and evaporated readily when the plates were removed from the developing tank. This phase was separated from the slower moving part of the solvent by a faint, yet distinct, yellow line which did not fade on drying.

The R_F values of P, V and S, relative to the demixion line, were approximately 0.87, 0.61 and 0.34 respectively. These R_F values, and the R_F value of the demixion line relative to the solvent front, varied according to the degree of saturation of the atmosphere in the developing tank. In a saturated atmosphere the demixion line did not move far enough up the plate for effective separation of P, V and S, while in an unsaturated atmosphere P was at the demixion line. The degree of saturation required for optimal separation can be readily determined from a few trial runs.

STONE AND BLUNDELL[2] found that the pure compounds, after separation by paper chromatography, could be determined to within ± 3 %, after correction for chromatographic losses. The gas chromatographic method of PEPPER *et al.*[3] gave agreement with the original composition to within 3 %. The results presented in Table I indicate that P, V and S may be determined with a greater accuracy by TLC than by either of the above two methods, agreement with the original composition being within 2 %.

Preliminary investigations showed that P, V and S could not be quantitatively extracted from an aqueous solution by two extractions with a small volume of chloro-

form. If, however, the aqueous phase is saturated with ammonium sulphate, all three phenolic aldehydes are readily removed. This method has been successfully employed for the quantitative extraction of P, V and S after the oxidation of lignified material.

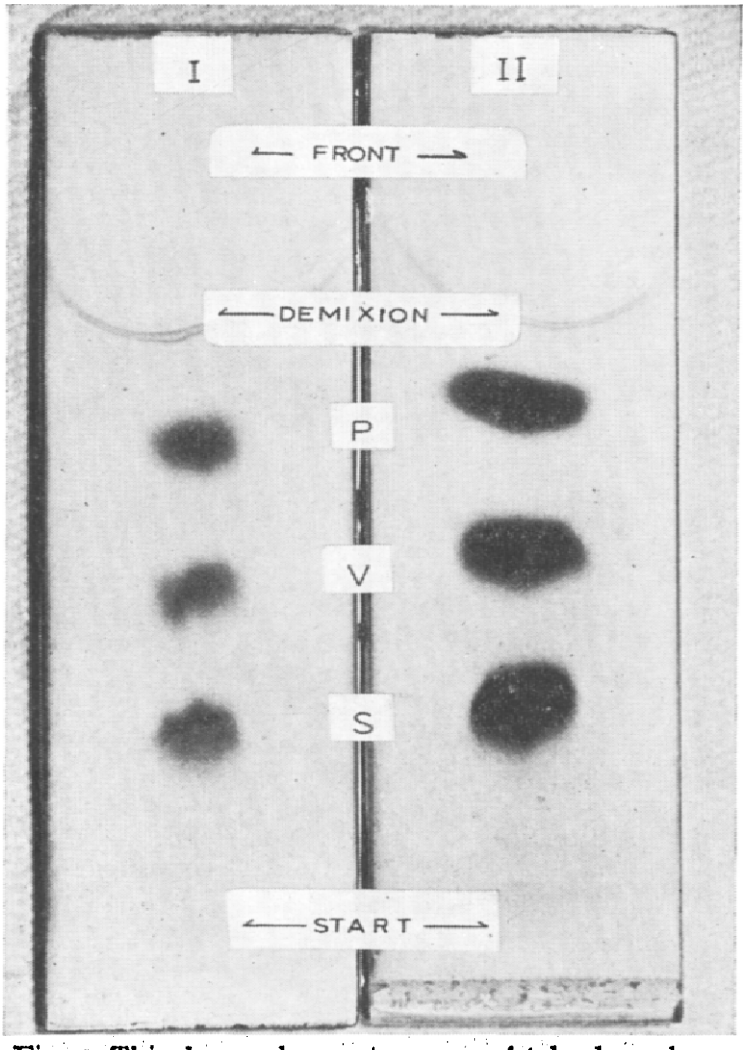

Fig. 1. Thin-layer chromatograms of *p*-hydroxybenzaldehyde (P), vanillin (V), and syringaldehyde (S) on silica gel G with *n*-hexane–isoamyl alcohol–acetic acid (100:16:0.25) as solvent. Plates sprayed with 2,4-dinitrophenylhydrazine. Plate I: 10 μg each of P, V and S. Plate II: 100 μg each of P, V and S.

The subsequent separation of these three substances by TLC provides a rapid and accurate method for their determination. It seems likely that this method could be adapted for the extraction of similar substances from a variety of sources.

SUMMARY

The separation of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde by thin-layer chromatography on HCl-washed silica gel G using a new solvent system, *n*-hexane–isoamyl alcohol–acetic acid (100:16:0.25), is described. All three of these phenolic aldehydes can be quantitatively extracted by chloroform from saturated

ammonium sulphate solution and quantitatively determined after separation on chromatoplates.

NOTE ADDED IN PROOF

Since the preparation of this article the quantitative TLC separation of P, V and S has been described by REALE[11]. The slightly low recovery of S reported by REALE could possibly be improved by acid-washing the silica gel.

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