Indian Inst. Sci., 64 (C), Mar. 1983, Pp. 1-9
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Separation of protocatechuate and homoprotocatechuate by paper chromatography and estimation of protocatechuate by colorimetry using a new $FeCl_3$ reagent

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Received on May 31, 1982: Revised on July 19, 1982.

Abstract

A new chromogenic reagent for orthodihydroxy phenolic compounds is prepared and its use in detection of protocatechuic acid, homoprotocatechuic acid and protocatechuic aldehyde after separation by paper chromatography is described. The reagent is also useful for the colorimetric estimation of protocatechuic acid.

Key words: Paper chromatography, protocatechuate, homoprotocatechuate, colorimetry.

1. Introduction

Phenolic compounds are ubiquitously distributed and occur frequently in plants of economic importance. Paper chromatography (PC) and thin layer chromatography (TLC) are methods of choice in qualitative analysis of phenolic substances. Recently, a quantitative approach to their analysis was possible using HPLC[1], but the analysis of phenolic compounds is rather general in nature and lacks specificity. A method for the estimation of orthodihydroxy compounds was earlier reported from this laboratory[2]. This method, though sensitive, had the same problem of being non-specific, reacting with a wide variety of orthodihydroxy compounds. Workers in this field have time and again pointed out this problem in quantitative analysis of phenolic substances[1,3]. A statistical method for choice of mobile and stationary phases in PC and TLC of phenolic compounds has been discussed[1]. It was stated that though for quantitative analysis of phenolic substances the technique of HPLC is being perfected, the preferred methods for their qualitative analysis are PC and TLC because of the wide range of

possibilities they offer in selecting appropriate mobile and stationary phase to get better chromatographic separation.

During our studies, we also faced a problem in separating a mixture of closely related phenolic substances by paper chromatography. These compounds were homoprotocatechuate and protocatechuate which moved close enough to appear as a single spot on paper chromatograms in various solvent systems which were tested. This problem was compounded by the non-availability of suitable spraying reagent that could impart different colours to these phenolic compounds. However, a combination of ammonium molybdate and ferric chloride was tested which gave different colour reactions for these two closely related catecholic substances. This finding has led not only to the development of a spraying reagent for the detection of protocatechuate, homoprotocatechuate and protocatechuic aldehyde but also to a colorimetric method for the estimation of protocatechuic acid.

In addition to the use of the reagent in qualitative analysis of certain important catecholic compounds, we also report here the application of this new reagent in quantitative analysis of protocatechuic acid.

2. Materials and methods

All the biochemicals used were from Sigma Chemical Co., except the following which were procured from other sources. *p*-Hydroxybenzaldehyde and 2,3-dihydroxybenzoic acid were from Koch-Light Laboratories and Aldrich Chemical Company, respectively. *m*-Cresol was procured from Sojuzhim Export, Moscow, and dihydrocaffeic acid from ICN Pharmaceuticals Inc., New York.

2.1. *Paper chromatography*

Paper chromatography was done on Whatman No. 1 filter paper (30 × 20 cm). The samples (40 μg of each compound in 40 μl of ethyl acetate) were applied directly on a line 2·5 cm from the bottom of the paper. The first spot (a mixture of protocatechuate, homoprotocatechuate and protocatechuic aldehyde) was 3 cm from the side of the paper and following three spots of individual compounds were at 4 cm intervals. Ascending elution in a close glass chamber was applied. A meter scale was used to measure R_f values of the spots.

2.2. *Solvent system*

Six solvent systems were tested in order to find those which give reasonable separation. The compositions (by volumes) of the solvents were as follows :

(1) Benzene : acetic acid : water (10 : 7 : 3)
(2) Isopropanol : ammonia : water (8 : 1 : 1)

(3) Ethanol : ammonia : water (18 : 1 : 1)
(4) Butanol saturated with 2% aqueous NH_4OH
(5) Butanol : pyridine : water (10 : 3 : 3)
(6) 2% formic acid.

2.3. *Development of chromatograms*

Development was continued until the solvent front had moved 26 cm from the spotting line on the paper. Subsequently, the paper was dried at room temperature (27 ± 2° C) and sprayed with chromogenic reagent.

2.4. *Preparation of solutions*

Solutions of all the chemicals and reagents used were prepared with deionised distilled water.

2.5. *Preparation of ferric chloride reagent*

One gram of ammonium molybdate was stirred in 70 ml of water till a sufficient quantity of it was dissolved and 5 ml of 10% ferric chloride solution was added dropwise when a lemon yellow precipitate appeared in the reaction mixture. Stirring of the solution was continued for ten minutes after the addition of the ferric chloride. The reaction mixture was brought to a volume of 100 ml by adding water and centrifuged at 27,000 g for 20 min to separate the precipitate formed. The golden yellow supernatant was filtered through Whatman No. 1 filter paper and the clear filtrate was the ferric chloride reagent which was stored at room temperature. The pH was found to be 1·8.

2.6. *Assay procedure*

To one millilitre solutions of protocatechuic acid (100 μg/ml) and homoprotocatechuic acid (100 μg/ml) equal volumes of molybdate reagent were added and shaken. The assay tubes were left for 60 min after colour development which took place immediately after the addition of the reagent. Homoprotocatechuic acid gave a light brown colour while protocatechuic acid formed bluish green colour complex. After addition of 1 ml of water to each test tube, spectra of the complex in the visible range were recorded in a Beckman model-26 spectrophotometer.

In a similar way, a calibration curve was prepared using standard concentrations of protocatechuic acid and the colour intensity was measured using filter No. 66 (630–690 nm) in a Klett Summerson Colorimeter.

2.7. *Recovery experiments*

In order to check the recovery of protocatechuate from the paper, two sets of five analyses were run. One set consisted of chromatographed protocatechuic acid containing 40 μg of the compound. After development of the chromatograms, the phenolic acid was located by UV fluorescence and the spot was eluted in the following manner: the area

of the paper corresponding to the spot was cut into small bits and placed into a test tube. The bits of paper were extracted with 2 ml of water by mixing in a vortex mixer for 2 min and were separated by centrifugation at a low speed to get a clear supernatant. This procedure of extraction gave a solution which would contain 20 μg/ml of phenolic acid if complete extraction occurred. These were run against unchromatographed solutions of protocatechuate obtained by diluting stock solution so that concentration in each test tube was 20 μg/ml. Both sets were treated exactly the same way and were run simultaneously.

A check on recovery of protocatechuate from plant, animal and bacterial extract was carried out. All extracts were prepared in 0·05 M sodium phosphate buffer, pH 7·0 by homogenising separately the leaves of *Tecoma stans* (15 g in 45 ml buffer) and rat liver (3 g in 9 ml buffer) in a Waring blender for 2 min and the resulting brei was filtered through two layers of cheese cloth and spun at 27,000 g for 15 min. The supernatants were used as extracts of animal and plant tissues. Five grams of bacterial cells (*Nocardia* sp.) were homogenised in 15 ml of buffer with glass powder using pestle and mortar and processed as described above and the resulting supernatant was used as bacterial extract.

A known amount of protocatechuate was added to the extracts (1 ml per assay tube) and after addition of 0·5 ml of 10% trichloroacetic acid (TCA) to each tube, extraction with 5 ml of ether was done by mixing for a minute and 2·5 ml of ether was pipetted out for evaporation. The residue was dissolved in 1 ml of water. Controls were also run which contained the same amount of phenolic acid in 1 ml of water and were treated similarly after addition of 10% TCA.

3. Results

3.1. *Colour reaction*

When the reagent was sprayed on the chromatograms it imparted a bluish green colour to spots corresponding to protocatechuic acid and protocatechuic aldehyde while the homoprotocatechuate spot turned dark brown.

3.2. R_f *values*

As evident from Table I, best separation of the closely related catecholic compounds, protocatechuic acid and homoprotocatechuic acid was achieved in solvent 6. Protocatechuic aldehyde separates well in all the solvent systems tested.

3.3. *Stability and spectral properties of the colour complex*

The spectrum of the colour complex of protocatechuic acid has two distinct peaks, while that of homoprotocatechuic acid has only one (fig. 1). The colour developed is stable for one hour and there is a 50% reduction in colour intensity after 12 h.

Table I

Chromatographic behaviour of phenolic compounds using various solvent systems

Sl. No.	Solvent system	Development time (mts.)	R_f values Protocatechuic acid	Homoprotocatechuic acid	Protocatechuic aldehyde
1.	Benzene : Acetic acid : Water (10 : 7 : 3)	180	0·14	0·13	0·26
2.	Isopropanol : Ammonia : Water (8 : 1 : 1)	720	0·12	0·17	0·54
3.	Ethanol : Ammonia : Water (18 : 1 : 1)	600	0·31	0·45	0·74
4.	Butanol saturated with 2% aqueous NH_4OH	630	0·02	0·04	0·35
5.	Butanol : Pyridine : Water (10 : 3 : 3)	600	0·28	0·26	0·90
6.	2% formic acid	105	0·47	0·71	0·58

3.4. *Specificity and effect of interfering substances*

As is evident from Table II, only catechol and protocatechuic aldehyde give the same colour reaction. The intensity of the colour given by protocatechuic aldehyde and protocatechuic acid is comparable, while that given by pyrocatechol is about half this value. The closely related compound, homoprotocatechuic acid, however, gives an intensity too insignificant to interfere with the assay reaction. None of the other hydroxy compounds listed in Table II give any colour with the $FeCl_3$ reagent, demonstrating the specificity of the $FeCl_3$ reagent.

Trichloroacetic acid interferes with the assay and addition of 5 mg of this acid to the assay mixture caused disappearance of the colour instantaneously.

.5. *Sensitivity*

he absorbance was a linear function of amount of protocatechuic acid at least in the inge of 10–100 μg (fig. 2). A solution of 10 μg/ml has an absorbance of 7 Klett units KU).

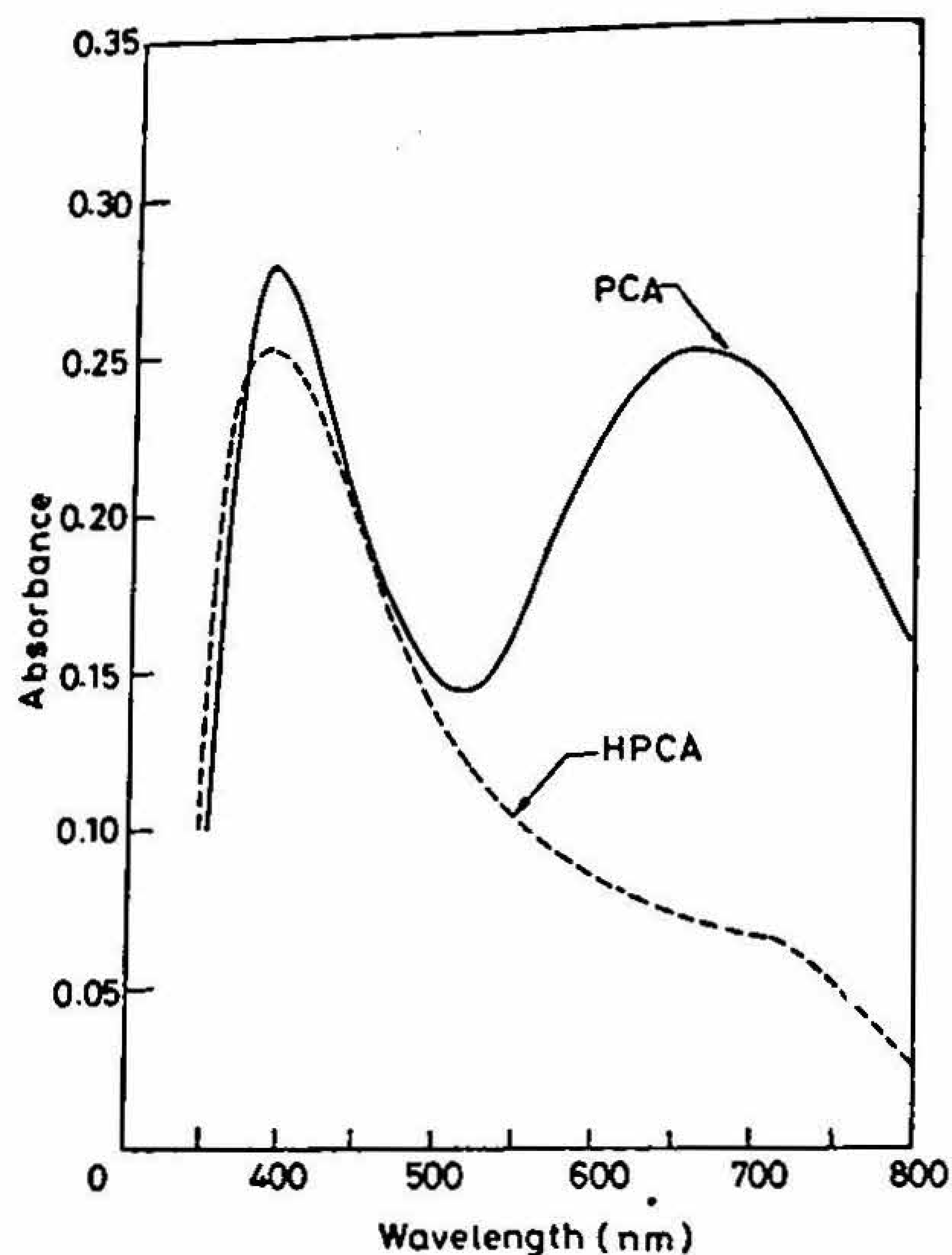

FIG. 1. Absorption spectra 60 min after the addition of ferric chloride reagent to a solution containing protocatechuic acid (PCA) (100 μg/ml) and homoprotocatechuic acid (HPCA) (100 μg/ml).

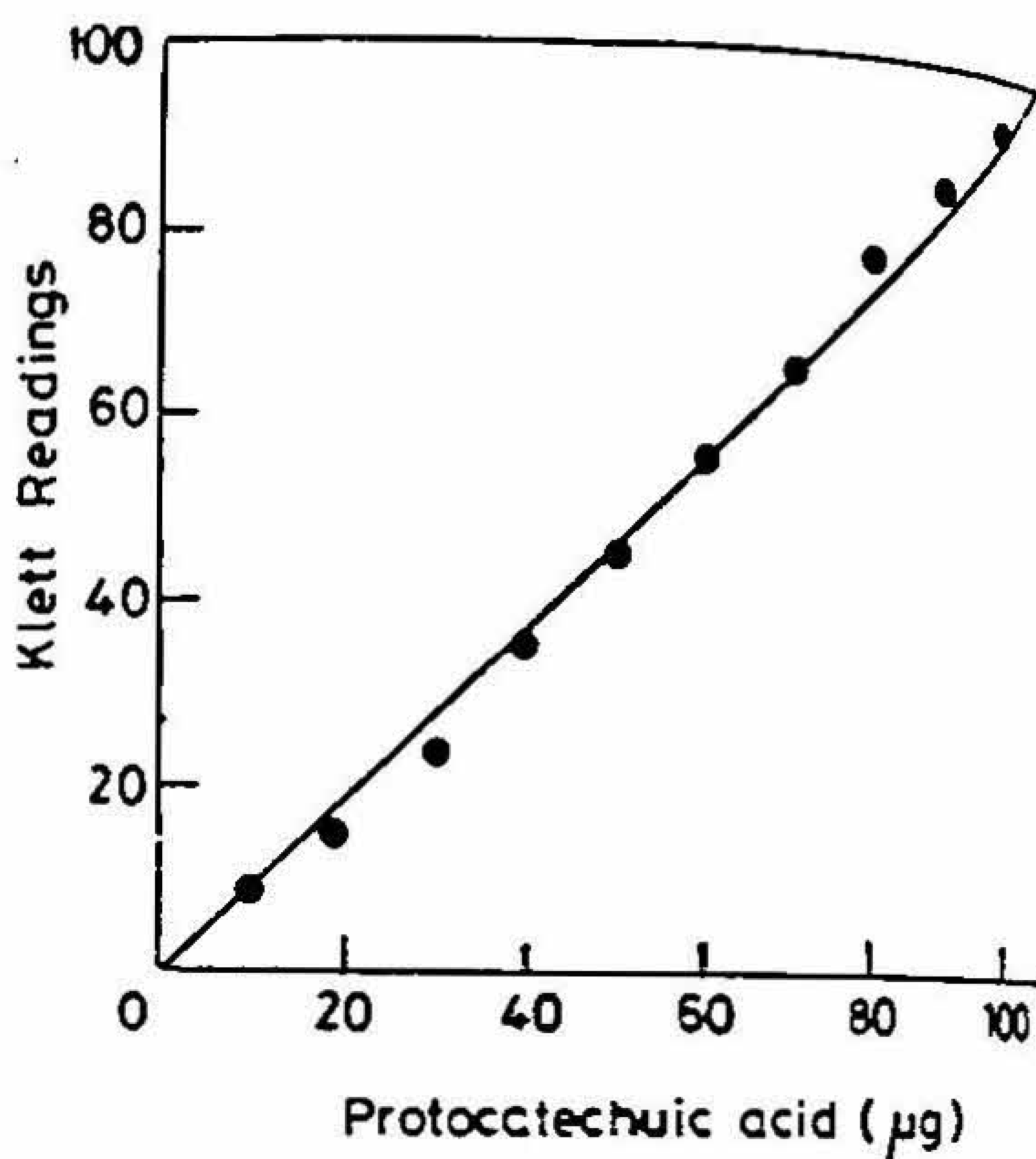

FIG. 2. The relation between absorbance and protocatechuic acid concentration after final colour development.

3.6. *Recoveries*

Table III shows recovery of protocatechuic acid from paper (106%), bacterial (89·56%), plant (87·5%) and animal extract (83·33%).

4. Discussion

A suitable chromogenic reagent has been developed which could distinguish between the closely related catecholic compounds, protocatechuic and homoprotocatechuic acids. We have successfully separated the compounds by paper chromatography and found, after testing various solvent systems, 2% formic acid (v/v) to be the most suitable for this purpose. Colour developed after spraying is stable and the spots are sharp and distinct. Thus the reagent can be used as a simple distinguishing test for protocatechuic and homoprotocatechuic acids in qualitative analysis.

Success of this reagent in qualitative analysis encouraged us to test its application in quantitative analysis. A method for estimation of catecholic compounds based on their complex formation with tungstate or molybdate reagent was first developed by Evans[1]

Table II

Specificity of the colour reaction

Substance tested (100 μg/ml)	Klett readings
p-Hydroxybenzoic acid	0
Protocatechuate	94
p-Hydroxyphenylacctic acid	0
Homoprotocatechuate	27
p-Hydroxymandelic acid	0
Protocatechuic aldehyde	91
Tyrosine	0
Dopa (3,4-dihydroxyphenylalanine)	4
m-Cresol	0
Adrenaline	0
Noradrenaline	0
Gallic acid	14
p-Hydroxybenzaldehyde	0
DL-Synephrine	0
2,3-Dihydroxybenzoic acid	0
2,5-Dihydroxybenzoic acid	12
p-Hydroxyphenylpropionic acid	0
Pyrocatechol	54
Caffeic acid	0
Dihydrocaffeic acid	0
Ferulic acid	0
Syringic acid	0
Vanillic acid	0

Table III

Recovery of protocatechuate from paper and cell-free extracts

Sl. No.	Source	Concentration	Klett units		Recovery (%)
			Mean*	Std. deviation	
1.	Stock solution	40 μg/spot	31·7	2·5	
	Eluted from paper		33·6	2·6	106·0
2.	Stock solution	100 μg/assay tube	90·8	3·7	
	Plant extract		79·4	3·3	87·5
3.	Stock solution	75 μg/assay tube	66·2	4·5	
	Rat liver extract		55·2	4·1	83·3
	Stock solution	50 μg/assay tube	44·5	3·2	
	Bacterial extract		39·8	3·7	89·6

Results of 5 determinations

and was modified by Nair and Vaidyanathan[2] for the determination of pyrocatechol. However, the modified method, though sensitive, does not have a narrow range of specificity and the reagent used gives the same colour with the compounds to which it is specific.

To the best of our knowledge this is the first colorimetric assay for catecholic compounds which has a very narrow range of specificity. The ferric chloride reagent is easy and simple to prepare and could be stored at room temperature for at least a month. The method could be employed in enzyme assays where protocatechuic acid is the product. Interference by trichloroacetic acid (if used for stopping the enzyme reaction) could be avoided by extracting the product into solvent ether, and by evaporating the solvent the product formed could be assayed. Protocatechuic aldehyde which also interferes with the assay could easily be eliminated upon ether extraction after acidification of the reaction mixture when both protocatechuic aldehyde and protocatechuic acid are taken up by the ether layer from which the acidic compound could be brought into bicarbonate solution (5%) leaving the neutral protocatechuic aldehyde in ether (neutral fraction). Some of the common phenolic substances present in plants like caffeic, ferulic, syringic acids, etc. have no effect on the colour reaction. Thus, the assay is also useful for direct estimation of protocatechuic acid in plant samples.

The sensitivity, narrow range of specificity and easy method of preparation of the reagent make this method suitable for enzymatic assays where protocatechuic acid is the product formed. We have also tested the effect of plant (leaves of *Tecoma stans*), animal (rat liver) and bacterial (*Nocardia*) extracts in recovery experiments. We could get a recovery which is quite high (Table III). This method also could be employed in direct estimation of protocatechuic acid in biological samples.

A check on interfering subtances should always be made by conducting recovery experiments before a quantitative analysis is done. Protocatechuic aldehyde's interference could be avoided as mentioned earlier by taking it up in neutral ether fraction, or the aldehyde could be separated chromatographically. As the recovery from the paper is complete (Table III), protocatechuate could be eluted and estimated after paper chromatography. The recovery from paper of 106% is not critical if an internal standard is used and its recovery is taken as 100%. Coupling the assay to paper chromatography is quite simple since 2% formic acid gives better separation in a short developmental time of 105 min (Table I).

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