A NEW SIMPLE SPECTROPHOTOMETRIC ASSAY OF PHENYLALANINE AMMONIA-LYASE

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ABSTRACT

A new spectrophotometric assay for the enzyme phenylalanine ammonia-lyase has been developed. The assay is simple and as sensitive as the conventional radioactive assay. Absorbance at 290 nm in toluene of *t*-cinnamic acid is the basis of this new assay. The relative merits and advantages of the assay are discussed.

INTRODUCTION

PHENYLALANINE ammonia-lyase (PAL) is a key enzyme in the phenolic metabolism of plants[1]. This enzyme has been purified to near homogeneity from several plant sources[2]. Unsuitable assay systems have often given rise to misleading conclusions about the properties and behaviour of this physiologically important enzyme[3,4]. An assay system which uses yellow colour reaction of cinnamic acid with potassium permanganate has been reported recently without being described in detail[5]. Even buffers used in the assay should be carefully chosen[6]. A few of the difficulties with hitherto known spectrophotometric assays have been reviewed by Camm and Towers[8]. Even the radioactive assay is not without certain pitfalls, such as interference by transaminase in crude enzyme preparations[9]. A modified radioactive assay based on the elimination of 3Pro-S proton of L-phenylalanine side chain by deamination reaction of PAL[10] is being used nowadays.[11]

The above discussion points out the need for the multiplicity of the PAL assays to suit the specific experimental conditions of the system under study. Hence, we now report a simple, new spectrophotometric assay for PAL in mint (*Mentha viridis* L.) leaves.

MATERIALS AND METHODS

t-Cinnamic acid, 2-mercaptoethanol, L-phenylalanine and polyvinylpyrrolidone were from the Sigma Chemical Co., Mo., U.S.A. Locally available Analar grade solvents were used. L-Phenylalanine^{14}-C-(U) was obtained from Bhabha Atomic Energy Research Centre, Bombay, India.

Enzyme preparation: About 50 g of leaves were homogenized in 150 ml of 5 mM Tris-HCl buffer, pH 8.5 containing 1.4 mM 2-mercaptoethanol. The resulting slurry was filtered through two layers of cheese cloth. The filtrate was spun at 12000 × g for 15 min. The supernatant was used as the crude enzyme.

Enzyme assays: PAL was assayed by the following methods:

Method A: In this method, the reaction mixture contained 0.05 M Tris-HCl buffer pH 8.8, 1.5 mM L-phenylalanine, 25,000 cpm of L-phenylalanine-^{14}C(U) (specific activity 351 mCi/mM), 50 mM 2-mercaptoethanol and 0.6 per cent soluble polyvinylpyrrolidone and 0.25 ml of the crude enzyme. This reaction mixture was incubated for 3 hr at 30°C. The reaction was stopped by 0.1 ml of 6N HCl. To this mixture 2 ml of toluene was added and shaken vigorously for 2 min. The toluene layer was separated and dried over anhydrous sodium sulphate. One ml of toluene was transferred to a scintillation vial containing PPO (2,5-diphenyloxazole)-toluene counting fluid and counted in a Beckman LS 100 scintillation counter. A parallel set of assays was done in a similar way except for the extraction of product which was done by using peroxide-free ether. The ether layer was dried over anhydrous sodium sulphate and evaporated and the residue was taken in a small volume of ethanol. After the addition of trace amounts of *t*-cinnamic acid, paper chromatography was performed in benzene : acetic acid : water (10 : 7 : 3) solvent system (organic phase). The area corresponding to *t*-cinnamic acid was cut and counted. In all the assays a reaction blank was used without the enzyme and this value (toluene soluble counts in phenylalanine -^{14}C(U) was subtracted from the enzyme reaction values.

Method B: Crude enzyme preparations cause considerable turbidity in the usual spectrophotometric assay and a modified new procedure of PAL assay was adopted. For this purpose a spectrum of cinnamic acid

was taken and the acid had considerable absorbance at 290 nm. A standard curve was prepared as follows: From stock solution aliquots containing graded amounts of cinnamic acid (10–100 nmoles) were taken, diluted to a constant volume of 0.25 ml with 0.05 M Tris-HCl buffer pH 8.8. To each of the test tubes 0.25 ml of 2N HCl was added followed by 2 ml of toluene, vortexed for 2 min, and centrifuged at a low speed to separate the aqueous from the toluene layer. One ml of the separated toluene layer was dried with a pinch of anhydrous sodium sulphate and was taken in a 1 ml cuvette and the absorbance at 290 nm was recorded. The standard reaction mixture contained the enzyme (0.1 ml), 20 mM L-phenylalanine and 50 mM Tris-HCl buffer pH 8.8. The assay was carried out at 30°C for 1 hr. The cinnamic acid formed was extracted in toluene and estimated as described above using the standard curve. In all the above experiments only freshly purified specimens of *t*-cinnamic acid were used to avoid yellow contaminants which develop on storage.

Identification of reaction product: The product formed by PAL reaction, under a given assay condition, was isolated by paper chromatography (Method A), with authentic *t*-cinnamic acid serving as a reference compound and its spectral absorption characteristics were noted.

Protein estimation: Protein estimation was done using the method of Lowry *et al*[12]. Interfering substances like β-mercaptoethanol (concentration same as used in the enzyme assay) and ether extracted phenolics from acidified aliquots taken for protein estimation were included in the blank cuvette and their interference in protein estimation was thus avoided.

RESULTS AND DISCUSSION

There are several spectrophotometric assays for PAL[13,14]. Some of the assays are direct and continuous. These assays are suitable when the activity in the crude enzyme preparations is high. Crude enzyme preparations with low enzyme activity pose several problems[15]; some of them being turbidity and high OD in the blank cuvettes (without the substrate). One way of eliminating them is the cumbersome procedure of using peroxide-free ether to extract cinnamic acid and dissolving the residue, after evaporation, either in acid[13] or alkali[14] and taking the OD at a suitable wavelength of the UV spectrum. Product extractions with ether are always tedious and time consuming.

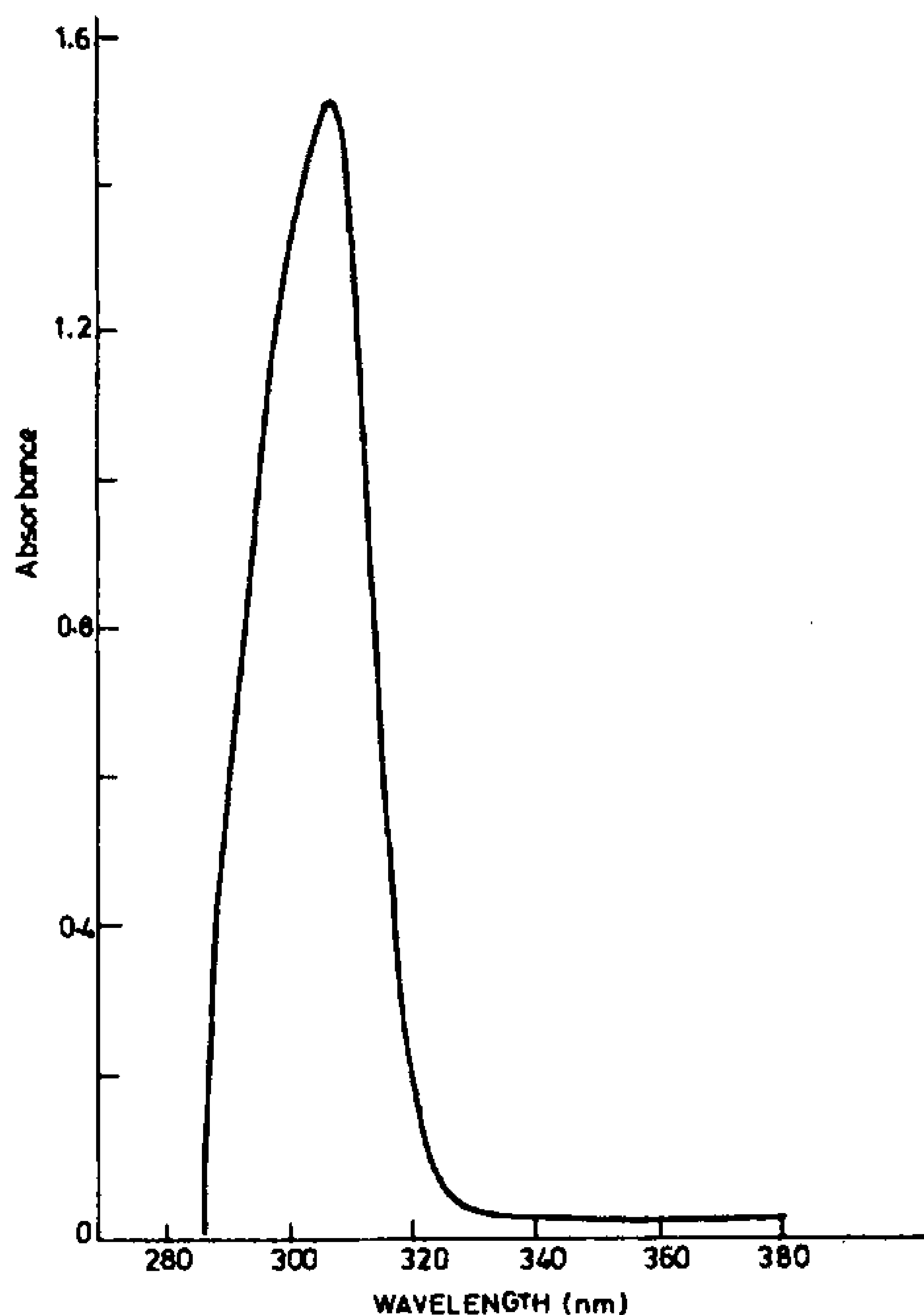

Figure 1. UV spectrum of *t*-cinnamic acid in toluene.

Table 1 *Comparison of the sensitivities of various spectrophotometric methods.*

Spectrophotometric assays	Wavelength of measurement (nm)	Molar extinction coefficient (ε)
O'Neal and Keller[13]	278	20,400
Zucker[14]	290	10,000
Present method	290 (in toluene)	20,000

Table 2 *Spectral characteristics of cinnamic acid in various solvents and specific activity of the crude enzyme.*

Solvent/ Methods of assay	λ_{max}	Specific activity*	
		Method A	Method B
Methanol	270	1.8	2
0.05 N NaOH	263	—	—
Toluene	315	—	—

* mU/mg protein, 1 Unit = 1 μmole of cinnamic acid/min at 30°C.

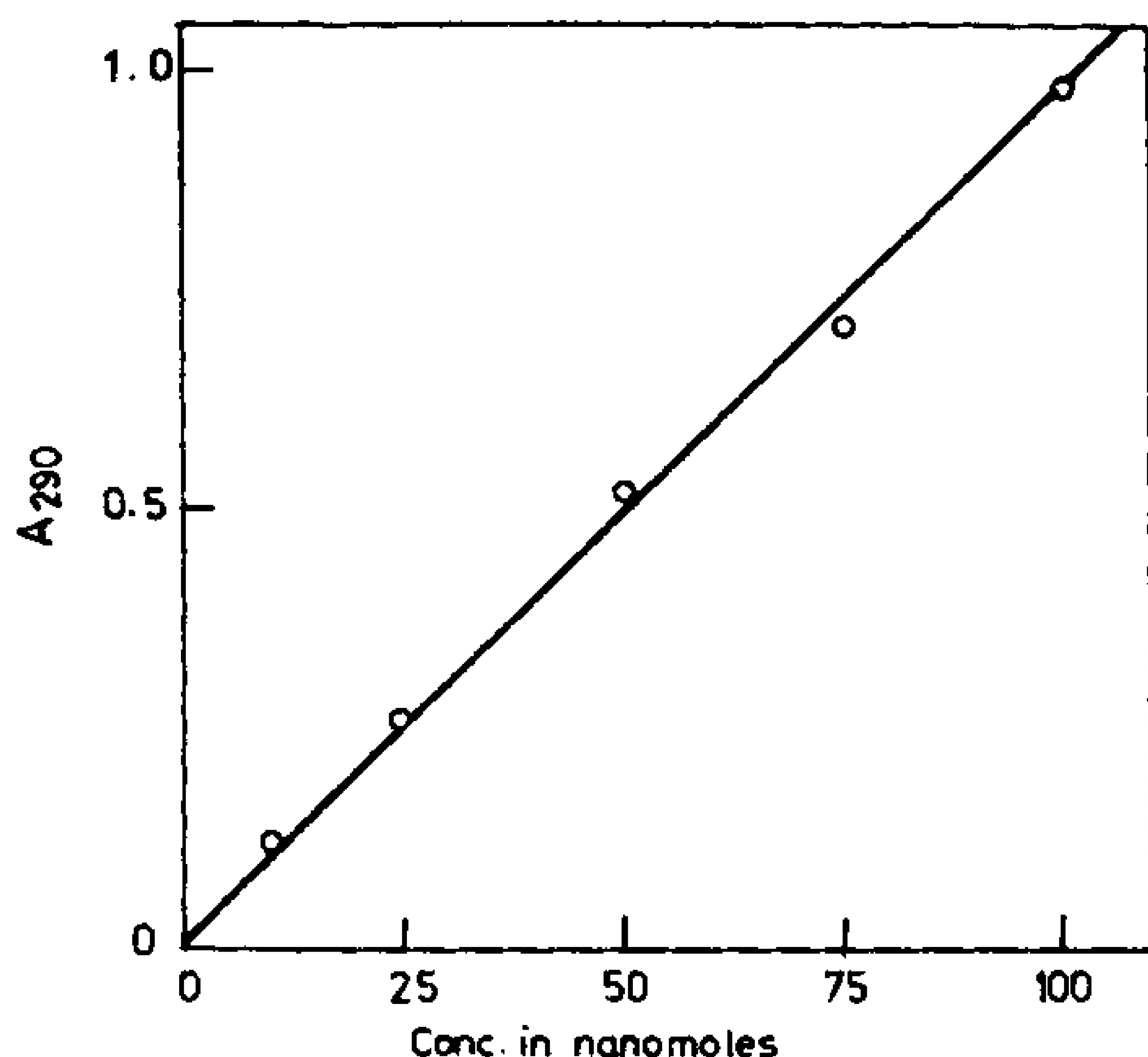

Figure 2. Standard graph for the estimation of *t*-cinnamic acid.

Literature survey gave us no indication of either λ_{max} or ε for cinnamic acid in toluene at 290 nm; we have got these values experimentally (tables 1, 2 and figure 1). The ε at 290 nm for cinnamic acid in toluene is 20,000. We report these values for the first time. Absorbance of cinnamic acid was a linear function of its concentration (20–200 nmolar). Table 1 gives a comparison of wavelength of measurement and ε obtained by our method and other reported ones. The values obtained by radioactive method and new spectrophotometric method are comparable (table 2) and possibly there is no transaminase interference. Moreover, we have recovered more than 80 % of toluene-soluble counts in the spot corresponding to *trans*-cinnamic acid on paper chromatography. The high boiling point of toluene makes for easier experimental handling while giving accurate and reproducible results.

As we have stated earlier the present method is no exception to observance of precautions for spectrophotometric assays. It is for these reasons we have isolated the reaction product, cinnamic acid and characterized it by UV spectroscopy. The assay has certain definite advantages over other spectrophotometric assays. It is simple, accurate and highly suitable for the crude enzyme preparations with low activity. We have used this assay successfully in the purification of PAL from the cytosol of mint leaves[15].

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