Journal of Chromatography, 604 (1992) 290–293
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 232

Short Communication

Determination of alkali-soluble phenolic monomers in grasses after separation by thin-layer chromatography

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(First received February 25th, 1992; revised manuscript received April 2nd, 1992)

ABSTRACT

A solvent system is described for the complete separation of ferulic acid, *p*-coumaric acid and vanillin on silica gel 60A K6F thin-layer plates. A spray reagent which distinguishes between these substances on a colour basis is also described. After separating the alkali-labile phenolic monomers in grass cell wall material on thin-layer plates, these substances were quantitatively eluted and assayed by means of a spectrophotometric procedure.

INTRODUCTION

In plant tissue, lignin is cross-linked to cellulose by means of low-molecular-weight phenolic acids [1,2]. During rumen digestion these links are broken and the phenolic monomers are released. Ferulic acid and *p*-coumaric acid seem to be the major components released from grasses [3].

At present, alkali-soluble phenolic monomers are receiving much attention from animal nutritionists as these compounds have been implicated in the poor digestibility of forage crops [4,5]. They have been shown to inhibit the growth of rumen bacteria and fungi [6,7] and decrease the digestion of structural carbohydrates [8–10]. A highly significant correlation was found between the digestibility of the cell walls from perennial ryegrass and the ferulic acid to *p*-coumaric acid ratio [5]. Alkali-labile phenolic monomers such as *p*-coumaric acid, ferulic acid and vanillin are usually separated by means of gas chromatography [11] or high-performance liquid chromatography [12].

Separation of these substances by means of thin-layer chromatography on silica gel plates is generally poor [13,14], thus making their quantitative analysis after thin-layer separation difficult. This prompted the authors to develop a solvent that would separate these compounds on silica gel plates. A spray reagent that could distinguish between compounds on a colour basis is also described. After separation, the individual phenolic acids could be eluted and accurately determined. The method was applied to the determination of alkali-labile phenolic monomers from the cell walls of several pasture grasses.

EXPERIMENTAL

Materials and reagents

All chemicals were of analytical-reagent grade. Standard solutions of vanillin (4-hydroxy-3-meth-

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0021-9673/92/$05.00

oxybenzaldehyde), ferulic acid (4-hydroxy-3-methoxycinnamic acid) and *p*-coumaric acid (4-hydroxycinnamic acid) were prepared by dissolving each compound (0.781 g) in ethanol (25 ml).

Cell wall material was isolated from kikuyu grass *(Pennisetum clandestinum)*, Italian ryegrass *(Lolium multiflorum)* and fescue *(Festuca arundinacea)* by the procedure of Van Soest and Wine [15].

A chromatographic solvent for the complete separation of vanillin, ferulic acid and *p*-coumaric acid on precoated silica gel 60A K6F (200 × 50 mm) plates was developed and consisted of hexane–ethyl acetate–isobutyl alcohol–2-propanol–acetic acid (21:2:1:1:0.1).

Phenolic substances on the chromatoplate were revealed by spraying with a reagent prepared as follows. Solution A was tin(IV) oxide (0.2 g) and iron(III) chloride (2 g) dissolved in 0.8 *M* hydrochloric acid (100 ml) and solution B was 2,4-dinitrophenylhydrazine (1 g) dissolved in 3 *M* hydrochloric acid (500 ml). Just before use, one part of solution A was mixed with three parts of solution B.

Extraction of phenolic monomers

Throughout the extraction process, care was taken to keep the samples away from sunlight. Grass cell wall material (0.5 g) was shaken in 1 *M* sodium hydroxide solution (20 ml) for 24 h at 20°C. The sample was vacuum filtered through a sintered-glass crucible and washed with distilled water (20 ml). The filtrate was acidified to pH 2.5 with hydrochloric acid and saturated with solid sodium chloride. The phenolic monomers were extracted with petroleum spirit (b.p. 80–100°C) (3 × 60 ml) using a separating funnel. The volume of the petroleum spirit extract was reduced to *ca.* 2 ml and then made up to 25 ml with ethanol.

Thin-layer separation

An aliquot (80 μg) of phenolic acid standard or phenolic monomer extract (0.4 ml) was applied as a band to an activated silica gel plate. The plate was developed with the proposed solvent in a chromatographic chamber (55 × 75 × 245 mm) for 40 min and subsequently dried at ambient temperature (21°C).

Identification

For qualitative identification of phenolic monomers, the plate was sprayed with the proposed spray reagent and heated (120°C, 10 min) to reveal the compounds. For quantitative analysis, the bands on the chromatogram were located by brief scanning under UV light.

Quantitative analysis

Each band was scraped off the plate into a centrifuge tube and the phenolic acid was dissolved in ethanol (2 ml). After centrifugation to remove the silica gel from the suspension, the phenolic acid

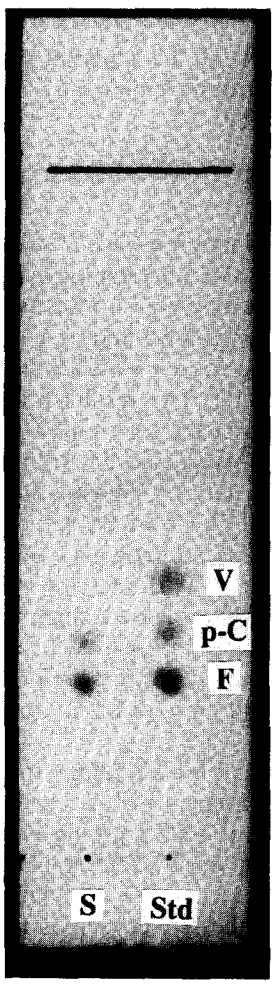

Fig. 1. Thin-layer chromatogram of phenolic monomers separated on a silica gel 60A K6F plate as described in the text. S = Phenolic compounds isolated from kikuyu *(Pennisetum clandestinum)* stem cell walls; Std = standard solution containing 10 μg each of ferulic acid (F), *p*-coumaric acid (p-C) and vanillin (V).

TABLE I

PHENOLIC MONOMER CONTENT OF GRASS CELL WALLS

Phenolic monomers were released from cell wall (CW) material, separated and determined as described in the text. Reported values represent the means of six analyses and the standard error of the mean.

Grass	Phenolic acid content (g/kg CW)		
	Ferulic acid	*p*-Coumaric acid	Total
Pennisetum clandestinum	6.2 ± 0.1	6.5 ± 0.3	12.7 ± 0.5
Lolium multiflorum	3.9 ± 0.3	12.1 ± 0.2	16.1 ± 0.3
Festuca arundinacea	3.7 ± 0.1	12.6 ± 0.1	16.3 ± 0.1

content in an aliquot (0.4 ml) of the supernatant solution was determined spectrophotometrically by the Folin–Denis procedure [16].

RESULTS AND DISCUSSION

The separation and determination of ferulic acid and *p*-coumaric acid are of considerable significance to ruminant nutritionists, as it has been shown that high *p*-coumaric acid concentrations and a low ferulic acid to *p*-coumaric acid ratio are associated with low forage digestibility [5]. The results presented in Fig. 1 show that complete separation of these substances and vanillin was achieved within 40 min using the proposed solvent system. No trailing of spots was observed. The mean R_F values (six replicates) of ferulic acid, *p*-coumaric acid and vanillin were 0.20 ± 0.02, 0.36 ± 0.01 and 0.46 ± 0.01, respectively. The R_F values varied slightly according to the degree of saturation of the chromatographic chamber. The chamber was therefore saturated with solvent for 30 min prior to the introduction of the plates.

The proposed spray reagent for revealing the positions of the phenolic monomers on the chromatoplate has the advantage of distinguishing between substances on a colour basis. The vanillin appeared as an orange spot directly after spraying, while *p*-coumaric acid and ferulic acid developed as light brown and dark mauve-brown spots, respectively, on heating the plate at 120°C. The detection limit of the spray reagent is *ca.* 0.2 μg.

The chromatoplate appears to lend itself to direct quantification of spots by fluorescence scanning. However, very satisfactory results were obtained by the spectrophotometric analysis of phenolic monomers after elution from the chromatoplates. The recoveries of ferulic acid, *p*-coumaric acid and vanillin were 100.2 ± 0.7%, 99.0 ± 0.7% and 100.1 ± 0.6% respectively (means of six recoveries).

The results in Table I show that reproducible results were obtained when the method was applied to plant cell wall material. It was further shown that the grasses analysed contained only ferulic acid and *p*-coumaric acid in significant amounts.

The new chromatographic solvent and spray reagent provide a rapid method for separating and identifying plant cell wall phenolic acids. These procedures, in conjunction with a spectrophotometric method for determining phenolic substances, offer an accurate technique for the determination of alkali-soluble phenolic monomers in plant cell wall material.

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