Journal of Chromatography, 134 (1977) 242–245

CHROM. 9833

Note

In situ reactions on silica gel thin layers in studies on plant oligosaccharides

ANNA LOMBARD, MARIA LUISA TOURN and MARIA BUFFA
Istituto di Chimica Farmaceutica e Tossicologica dell'Università di Torino, Turin (Italy)
(Received November 26th, 1976)

The technique involving *in situ* reactions is useful in chromatographic studies, particularly when dealing with microgram amounts of compounds, alone or in complex mixtures. The applications of *in situ* reactions on thin layers described since 1965 have been reviewed by Dallas[1]; earlier work on paper and thin layers was surveyed by Kirchner[2].

In carbohydrate research, *in situ* reactions have been applied on both paper and thin layers. Bacon and Edelman[3] treated sugars with invertase on paper between two-dimensional chromatographic developments. A two-dimensional chromatographic development interspersed with a reaction on paper was also employed by French *et al.*[4] in structure analysis of starch oligosaccharides. Wohnlich[5] reported a technique of enzymic hydrolysis on paper simultaneously with chromatographic development. Kartnig and Wegschaider[6] made use of acid hydrolysis on silica gel layers in the identification of the sugar moiety of glycosides.

This paper describes the application of the technique of *in situ* acid and enzymic hydrolysis on silica gel thin layers in studies on the composition and structure of plant oligosaccharides.

EXPERIMENTAL

Reference sugars were obtained from commercial sources. The sample solutions contained 5 mg of sugar in 1 ml of 70% ethanol; 2 μl were spotted on the plate.

Oligosaccharides were isolated from leaves of *Mentha arvensis* L. var. *parietariaefolia* Beckeck by extraction with hot 70% ethanol[7] followed by fractionation on Kieselgel G (Merck, Darmstadt, G.F.R.) layers with chloroform–acetic acid–water (3:3.5:0.5)[8] (3–5 ascents at 28°) and elution of the separated saccharides from the layer with 70% ethanol.

According to the classification of Dallas[1], the method of *in situ* hydrolysis that we applied was of the type O/D/AL/X_2SRS or O/D/AG/X_2SRS, *i.e.*, a method in which the reaction is intended, defined and applied between two developments and the reagent is a spray or a vapour.

For the *in situ* analysis, standard 20 × 20 cm plates with a 0.25 mm layer of Kieselgel G were used; 5–10 μg of each component were spotted on the plate. The same eluent (see above) was used in both directions; the multiple ascent technique was employed for better resolutions.

Acid hydrolysis

The hydrolysis was obtained by spraying the layer with 0.25–1 *N* hydrochloric acid and heating the plate in an oven at 80–100° for 30 min. Constant humidity conditions were ensured by covering the layer with a glass plate separated by strips of cardboard and held in place with adhesive tape (S chamber). An alternative procedure consisted in heating the plate at 90–100° for 30 min in the presence of hydrogen chloride vapour in a glass tank. At the completion of hydrolysis, excess of hydrogen chloride was eliminated under vacuum.

Hydrolysis with β-fructosidase

The hydrolysis was carried out by spraying with a 0.01% solution of β-fructosidase (from yeast, Boehringer, Mannheim, G.F.R.) and maintaining the plate for 30 min at 37° under constant humidity. The layer was then dried in a stream of hot air to stop enzyme action.

In both instances, parallel plates were run without hydrolysis. Reference sugars were either spotted on the plate before elution in the second dimension or added to the initial spot. An unhydrolyzable sugar (*e.g.*, a monosaccharide) was added to the initial spot as a marker in order to define the diagonal line on which unaltered compounds are located following two-dimensional elution with the same eluent. This permitted a check to be made of whether the hydrolysis had taken place, especially in the case of higher DP (degree of polymerization) saccharides.

The spots were rendered visible with various spray reagents, such as diphenylamine aniline phosphate (DAP), thiobarbituric acid (TBA) and triphenyltetrazolium chloride (TTC). Single and multiple detection techniques were employed[9].

RESULTS AND DISCUSSION

The *in situ* method of hydrolysis was used in the analysis of two oligosaccharides from *Mentha parietariaefolia* leaves. These components migrated in the region of trisaccharides and tetrasaccharides, on silica gel layers under the conditions described above. They were both non-reducing to TTC and were TBA-positive; their hR_G values were in the range 30–33 and 11–14*. On total acid hydrolysis (with 1 *N* sulphuric acid at 100° for 1–2 h), both gave glucose, galactose and fructose. Their chromatographic behaviour was identical with that of pure raffinose and stachyose[7].

On *in situ* partial hydrolysis, the trisaccharide component yielded fructose and a disaccharide identical with melibiose (Fig. 1a). Invertase hydrolysis also gave fructose and melibiose. The same products of hydrolysis were obtained from a pure sample of raffinose by *in situ* acid and enzymic hydrolysis under the same conditions.

Partial acid hydrolysis of the tetrasaccharide component gave fructose and a trisaccharide that did not contain fructose, identical with a sample of manninotriose (Fig. 1b). The latter was prepared by partial acid hydrolysis of authentic stachyose. Enzymic hydrolysis also cleaved fructose from the molecule of the tetrasaccharide. The behaviour of the tetrasaccharide was identical with that of stachyose in parallel runs.

* The hR_G (G = glucose) values were calculated after three ascents of the eluent.

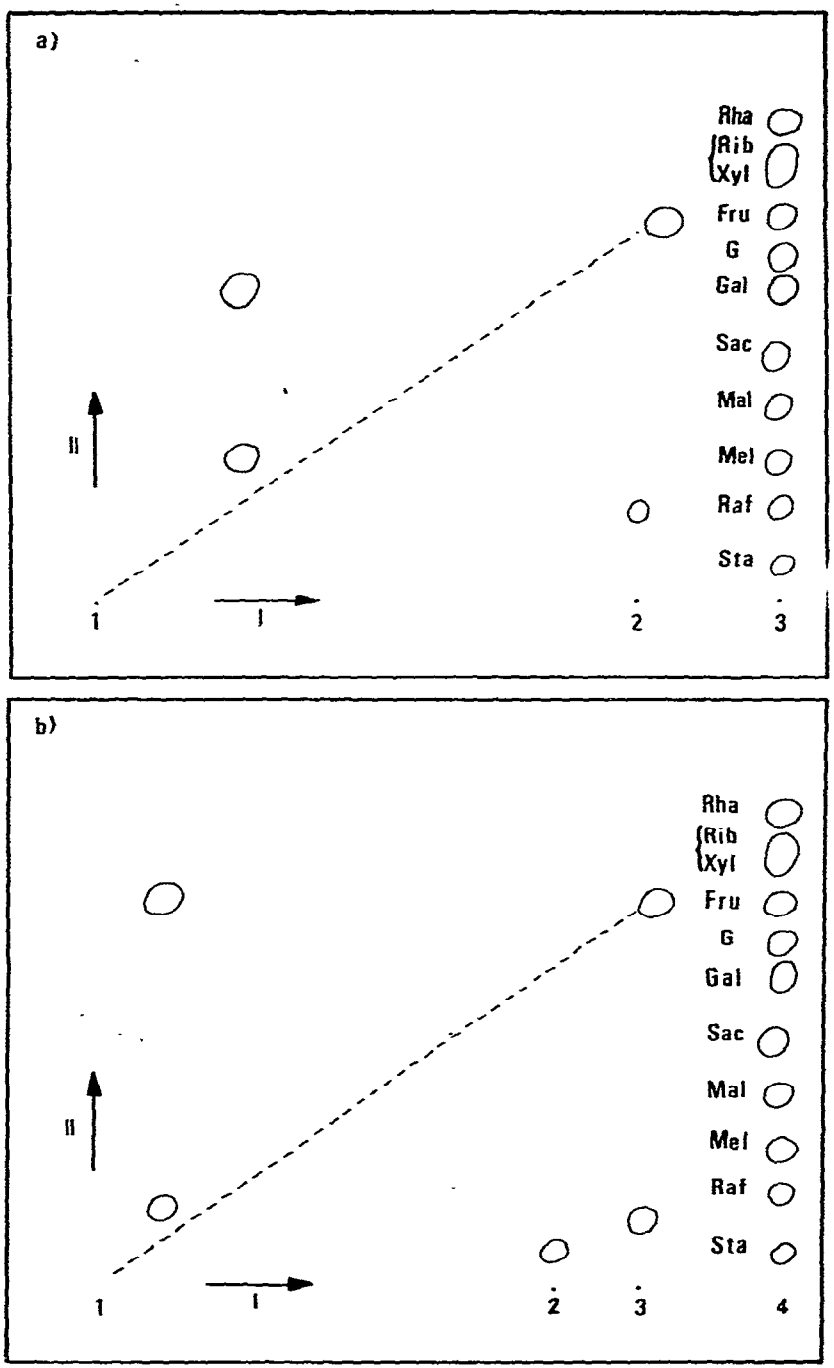

Fig. 1. *In situ* acid hydrolysis (0.25 *N* HCl, 80°, 30 min) of *Mentha* leaf oligosaccharides on a Kieselgel G layer with the solvent system chloroform–acetic acid–water (3:3.5:0.5); 3 ascents at 28°. The spots were detected with DAP. (a) Analysis of the trisaccharide. 1 = Trisaccharide + fructose (marker); 2 = trisaccharide (spotted before elution in the second dimension); 3 = sample sugars (rhamnose, ribose, xylose, fructose, glucose, galactose, saccharose, maltose, melibiose, raffinose, stachyose). (b) Analysis of the tetrasaccharide. 1 = Tetrasaccharide + fructose (marker); 2 = tetrasaccharide (spotted before elution in the second dimension); 3 = manninotriose; 4 = sample sugars [as in (a)].

On the basis of these results, the chromatographic identification of the two saccharides from *Mentha* leaves as raffinose and stachyose could be confirmed.

The method of *in situ* hydrolysis can give interesting information about the composition and structure of hydrolyzable compounds. It can be applied to microgram amounts of sample, alone or in mixtures. Other hydrolyzable substances present in the sample must be separable from the component under study by chromatography in the first dimension.

As the development is two-dimensional, the limits of detection are slightly lower than those found with the various reagents following one-dimensional development[9].

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