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QUANTITATIVE DETERMINATION OF MONO-, DI- AND TRISACCHARIDES BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A thin-layer chromatographic (TLC) system for determining simultaneously up to eleven carbohydrates commonly found in food chemistry is described. The TLC plates, pre-coated with silica gel, are developed three times in the same direction with a mixture of acetonitrile and water before dipping them in a solution containing diphenylamine. Quantitation is effected with a chromatogram spectrophotometer connected to a programmable chromatography data system.

INTRODUCTION

The quantitative determination of carbohydrates in the field of food chemistry can be performed by several methods. The most common approaches are enzymology, gas chromatography, column chromatography, thin-layer chromatography (TLC) and measurement of reducing saccharides with copper salts[1]. Today, enzymatic methods are used mainly for determining glucose, fructose, sucrose, lactose, galactose, maltose and raffinose[2,3]. These methods are highly specific but do not allow the simultaneous determination of a mixture of different carbohydrates.

In contrast to gas–liquid chromatography[4–6], column and thin-layer chromatography need no derivatization of the sample prior to analysis. In column chromatography, especially in high-performance liquid chromatography, the separation is performed mainly on supports with aminoalkyl groups chemically bonded to a stationary phase[7–10]. In contrast to these methods, TLC is a simple technique used almost in every analytical laboratory and used extensively in the separation of sugars. Many methods have been published in the last two decades, indicating the difficulty of separating these similar compounds. Impregnated silica layers[11,12], cellulose layers[13,14] and pre-treated alumina supports[15] have been proposed.

The aim of this work was to obtain a reliable TLC system for separating the carbohydrates commonly found in foodstuffs. After numerous efforts, the approach of Siegenthaler and Ritter[16] was modified to the needs of a quantitative and simultaneous determination of two trisaccharides (raffinose and melecitose), three disaccharides (lactose, maltose and sucrose), three hexoses (galactose, glucose and fructose) and three pentoses (xylose, rhamnose and 2-deoxyribose).

EXPERIMENTAL AND RESULTS

Principle

Aqueous extracts are separated on pre-coated silica gel plates with water–acetonitrile by developing three times in the same direction. The compounds are made visible by dipping the plate in a reagent containing diphenylamine, followed by densitometric (reflectance) comparison with co-chromatographed external standard.

Apparatus and reagents

Aluminium foils pre-coated with silica gel 60 (Art. 5553; Merck, Darmstadt, G.F.R.) or similar pre-coated glass plates (Art. 5715; Merck) were used, together with 1-μl microcapillaries. A chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.) and an SP 4000 integrating system (Spectra Physics, Santa Clara, Calif., U.S.A.) were employed.

Acetonitrile (pro analisi grade) was obtained from Merck and 85 ml were mixed with 15 ml of water to give the developing solution. The dipping solution consisted of 12 g of diphenylamine and 12 g of anilinium chloride, dissolved in 1000 ml of methanol and 100 ml of concentrated phosphoric acid (86%). The solution is usable for several weeks if stored in a dark bottle.

Standards

Normally the following saccharides were co-chromatographed as reference substances: raffinose, melecitose, lactose, maltose, sucrose, galactose, glucose and fructose. For special purposes the pentoses xylose, rhamnose and 2-deoxyribose were used in the same test solution. Three solutions of each sugar with different concentrations (4.0, 2.0 and 0.5 mg/ml) were prepared. The stability of the standard solution can be considerably improved if a small crystal of thymol is added and if it is stored in a freezer.

Samples

All of the above carbohydrates are readily soluble in water, and a simple aqueous extract normally is the only sample preparation needed[17]. Sometimes it may be necessary to precipitate proteins that interfere in the chromatography[18]. The saccharide content per spot on the plate should be within the calibration range (0.5–4 μg per spot). The volume spotted must be constant, preferably 1 μl.

Chromatography

The developing tank is conditioned for 10 min before developing. Each sample and standard is spotted in duplicate and the applied spots must be completely dry before development, which is carried out three times for 60 min at 21° in the same solvent and tank. Between the developments the silica gel layer must be dried for 20 min with a temperature of 40–45° on the surface of the plate, for instance with a hair dryer and at a distance of *ca.* 40 cm.

After the third development the thin-layer plate is dried again, either as mentioned above or at 100° for 2 min. The cooled plate is immersed completely for a moment vertically in the dipping solution by holding it at the side of the starting line. After this treatment, the plate is drained vertically for 1 min before it is dried

between two pieces of blotting-paper for a further 1 min. Finally, the spots are revealed by heating the horizontal plate for 10–15 min at 100–120°. Fig. 1 shows an example of a chromatogram obtained by this method.

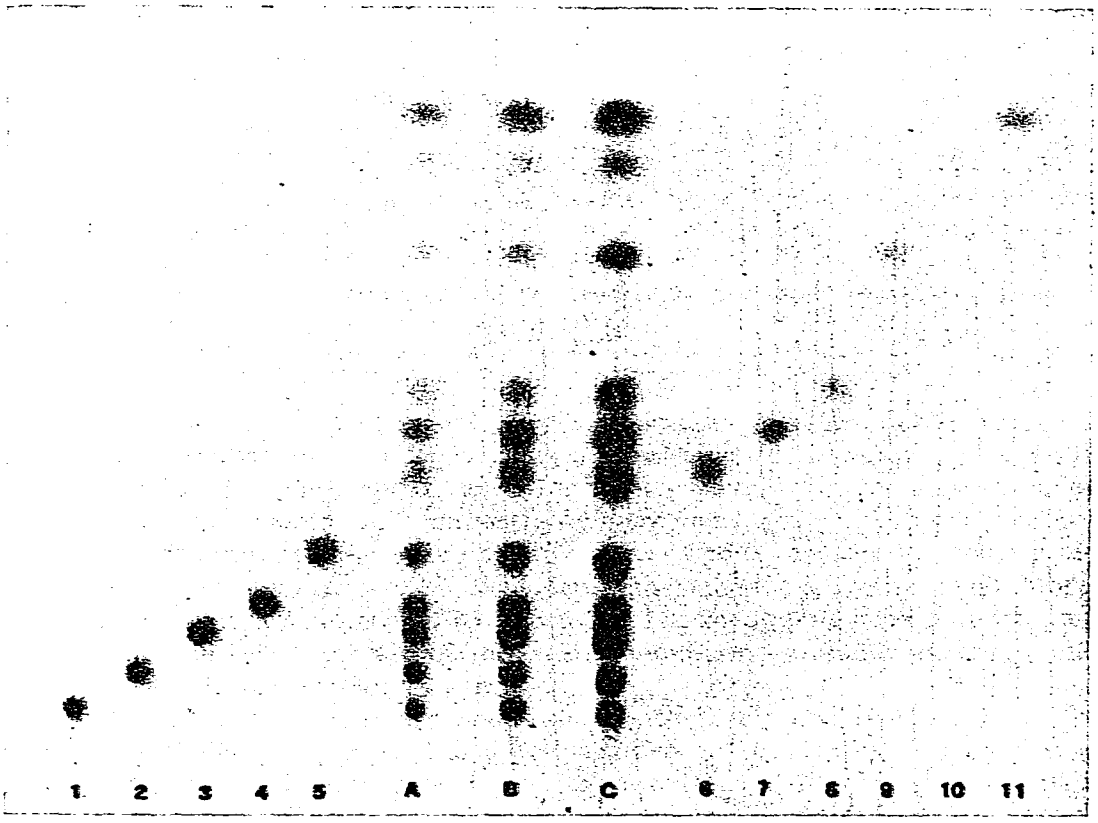

Fig. 1. TLC of 11 saccharides (for conditions see text): (1) raffinose; (2) melecitose; (3) lactose; (4) maltose; (5) sucrose; (6) galactose; (7) glucose; (8) fructose; (9) xylose; (10) rhamnose; (11) 2-deoxyribose. A, B and C = 0.5, 2 and 4 μg per sugar (mixture), respectively.

If samples with large differences in the amounts of lactose and maltose have to be analysed, it was found to be useful to add 2% glacial acetic acid to the developing solvent, but to the detriment of the separation of glucose and fructose. On the other hand, the separation of glucose and fructose can be improved by impregnating the plate with a solution of 0.2 *M* phosphate buffer (pH 6.8). The plate must be dried for 30 min at 110° before use with acetonitrile–water (85:15), but the lactose and maltose are no longer separated in this system.

Quantitative determination

The chromatogram spectrophotometer was used under the following conditions: beam path arrangement: monochromator–sample, with tungsten source; wavelength: 420 nm; slit width: 0.3 mm; and scanning stage speed: 50 mm/min.

The TLC densitometer is connected directly to a programmable SP 4000 chromatography data system. A typical densitometric scan is shown in Fig. 2. Retention

times of saccharides relative to sucrose ($R_F \approx 0.33$) are as follows: raffinose, 0.28; melecitose, 0.43; lactose, 0.62; maltose, 0.78; galactose, 1.33; glucose, 1.51; fructose, 1.59; xylose, 2.24; rhamnose, 2.62; and 2-deoxyribose, 2.85.

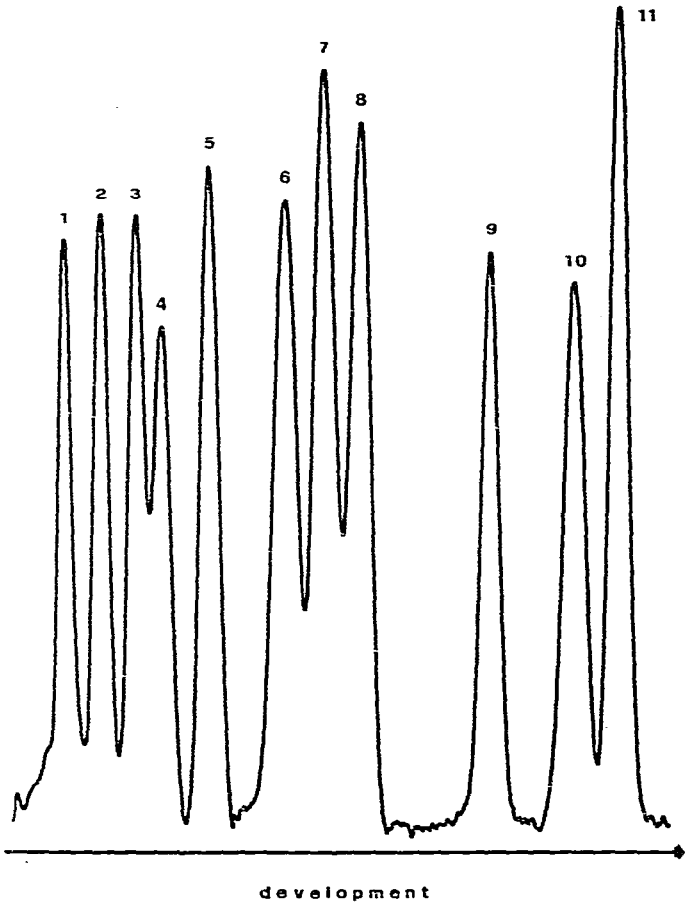

Fig. 2. Reflectance curve for 11 saccharides (4 μg each). For conditions, see text. Identities of saccharides as in Fig. 1.

DISCUSSION

The proposed method is satisfactory for the quantitative densitometric determination of most carbohydrates commonly found in foodstuffs. It is important that all of the described conditions are met in order to avoid substantial errors. The relative standard deviation for single measurements varies between 1.3% and 3.2%. Table I shows values obtained by spotting the same carbohydrate mixture several times on the same TLC plate. By scanning the same track on the plate eight times, deviations between 0.7% and 1.1% (Table II) produced by the densitometric system and differences in peak area measurement were found (exception: 2.0% standard deviation for raffinose, with the smallest R_F value). As the peak area of both standards and samples have similar errors, the total relative standard deviation is greater than

TABLE I

PEAK AREAS OBTAINED BY SPOTTING THE SAME CARBOHYDRATE MIXTURE (2 μg OF EACH SACCHARIDE) SEVERAL TIMES ON THE SAME PLATE

Measurement No.	*Integrated peak area (integrator counts)*							
	Raffinose	*Melecitose*	*Lactose*	*Maltose*	*Sucrose*	*Galactose*	*Glucose*	*Fructose*
1	17148	19432	17702	10151	20537	20701	22946	21099
2	17272	18716	17459	10348	21661	20352	22774	20194
3	18122	19647	17847	10989	20944	20757	22611	21053
4	17382	18734	17065	10794	21002	20325	21773	20688
5	18216	19251	16760	10974	20871	20236	21116	20469
6	17467	18892	19979	10382	21011	20585	22811	19885
7	18150	19425	17815	10851	21849	20954	22133	20153
Mean	17680	19157	17375	10641	21125	20559	22309	20506
Relative standard derivation (%)	2.6	2.0	2.5	3.2	2.2	1.3	3.0	2.3

4% owing to cumulation of errors; however, by applying the double evaluation method as described under *Chromatography*, the standard deviation of the result decreased below 3%. The standard deviation was decreased by up to 40% by dipping the plate in diphenylamine solution instead of spraying.

For economic reasons a dipping tank made of polypropylene was constructed (20 × 25 × 1 cm; cost *ca.* Sfr. 80; Semadeni, Ostermundigen, Switzerland). Moreover, the sensitivity was improved by a factor of two by dipping instead of spraying the plate, which led to the use of the small amounts of substance described and also improved the separation.

As in TLC the calibration graph is not linear[19,20], it is advisable to evaluate the results graphically. The SP 4000 data system offers the possibility of a multi-level calibration using several concentrations, but linear interpolation can produce additional errors of up to 5% between the calibration points. The graphs can be linearized with a parabolic approximation of $A^2 \doteq f(c)$ or a two-fold logarithmic approximation

TABLE II

PEAK AREAS OBTAINED BY SCANNING THE SAME TRACK ON THE PLATE EIGHT TIMES

Measurement No.	*Integrated peak area (integrator counts)*							
	Raffinose	*Melecitose*	*Lactose*	*Maltose*	*Sucrose*	*Galactose*	*Glucose*	*Fructose*
1	20441	23228	20461	13616	22747	20830	24686	25142
2	20994	23354	20733	13646	22653	20938	24598	24898
3	20286	23190	20495	13677	22648	20676	24550	25185
4	21220	23547	21122	13689	22775	20914	24214	25177
5	20483	23187	20832	13437	22589	20625	25058	24493
6	21033	23340	20619	13571	22476	21050	24455	25265
7	20978	23340	20681	13735	22522	20861	24291	25314
8	20122	23020	20482	13691	22316	20842	24701	25074
Mean	20695	23276	20678	13633	22591	20842	24569	25069
Relative standard deviation (%)	2.0	0.7	1.1	0.7	0.7	0.7	1.1	1.1

of $\log A = f(\log c)$, where A is the area enclosed by the reflectance curve and c is the concentration. This approach has the disadvantage of being more time consuming if an on-line calculator is not used. An important consideration in obtaining the precision mentioned is the integration parameters. The best results were obtained by resolving the sucrose peak as the baseline separated area and when saccharides behind and in front of this reference peak were integrated as fused peaks.

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