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THERMAL–ULTRAVIOLET METHOD FOR DIFFERENTIAL DETECTION AND RECOVERY OF ORGANIC COMPOUNDS IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Extending work on the detection of organic compounds in thin-layer chromatography using the thermal–ultraviolet method, the differential detection of sugars based on a temperature-dependent characteristic pattern revealed under UV and visible light is reported. Specific colours related to structural changes have been observed under UV light. High recoveries of ^{14}C- and also ^{3}H-labelled compounds after thermal–UV detection have been obtained. The detection of lipids and the determination of the sensitivity of the method for these compounds and carbohydrates are described. Results comparable to those in sulphuric acid-based methods have been obtained. In addition, the method has the advantage of a very low background even after prolonged periods of heating at high temperatures. The detection, differentiation and recovery of several methyl glucosides is also reported.

INTRODUCTION

The techniques currently used for the detection of organic substances in thin-layer chromatography (TLC) involve the treatment of the plates with various reagents, which generally implies the destruction of the sample. However, in many instances it may be necessary to recover the sample and, with labelled compounds, to measure the radioactivity after detection. Thus, it would be desirable to find a general method that is able to detect a wide range of different substances with the possibility of a high recovery of the sample.

Recently a thermal–UV method was proposed for the detection of organic compounds in paper chromatography based on the appearance of dark spots on the chromatogram[1]. The sensitivity of the detection procedure was significantly increased by examination under UV light. The successful detection of substances such as N-acetylamino acids, which are difficult to detect by other techniques, indicated the general applicability of the method[2]. This paper describes the application of the thermal–UV method to the detection and characterization of sugars and lipids in TLC.

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The results obtained revealed a differential detection of sugars based on a temperature-dependent characteristic pattern observed under UV and visible light and specific colours for some substances under UV light. The detection of lipids can be accomplished as easily as for carbohydrates and the sensitivity for the two types of substances is comparable to those of sulphuric acid-based methods.

For various ^{3}H- and ^{14}C-labelled compounds almost complete recoveries were achieved after detection in a given temperature range.

EXPERIMENTAL

Materials

Glass-based silica gel 60 thin-layer plates (20 × 20 or 5 × 20 cm, 0.25 mm thick) were purchased from Merck (Darmstadt, G.F.R.).

[U-^{14}C]Glucose (285 Ci/mol), [6-^{3}H]glucosamine (10 Ci/mmol) and [U-^{14}C]mannose (217 Ci/mol) were purchased from New England Nuclear (Boston, MA, U.S.A.).

Glucosamine, glucose, mannose, fructose, galactose, lactose, sucrose, gentiobiose, cellobiose, maltose, L-fucose, trehalose, raffinose, dolichol and cholesterol were reagent-grade materials from Sigma (St. Louis, MO, U.S.A.). β-Sitosterol, geraniol and squalene were purchased from K & K Labs. (Plainview, NY, U.S.A.). Kojibiose was obtained from Koch-Light (Colnbrook, Great Britain). Nigerose was a gift from Dr. Rodolfo Ugalde of this Institute. 2,4-Dimethylglucose, 3,4-dimethylglucose and 4,6-dimethylglucose were obtained from Supelco (Bellefonte, PA, U.S.A.).

Analytical procedures

Total brain lipids were extracted from the brain of a 5-day-old Wistar rat by the method of Folch *et al.*[3]. The lower phase was dried under a stream of nitrogen, resuspended and chromatographed as described previously[4,5]. Lipids corresponding to one fifth of the total extracted were spotted.

The following solvent systems were used: (A) benzene–acetone–ammonia solution–water (50:200:1.35:1); (B) *n*-butanol–pyridine–water (6:4:3); (C) ethyl acetate–*n*-hexane (1:9); (D) chloroform–methanol–water (70:30:4); (E) *n*-hexane–diethyl ether–acetic acid (40:60:2.3).

For routinely heating the plates, a conventional electric oven was used. For a more precise determination of the temperatures, a Packard Model 804 gas chromatograph oven equipped with a Model 873 temperature controller was used. The plates (cooled or hot) were observed under visible and UV light (366 nm).

Radioactivity was measured using toluene–PPO (2,5-diphenyloxazole) in a Beckman Model 8100 scintillation counter.

Radiochromatograms were obtained using a Packard Model 7200 radioactivity scanner.

Methylation of saccharides

Methylation of [U-^{14}C]glucose, cellobiose, gentiobiose, kojibiose and nigerose was carried out according to Hakomori[6]. The permethylated disaccharides were hydrolysed as described previously[7]. The following compounds were obtained by this

procedure: 2,3,4,6-tetramethyl[U-^{14}C]glucose, 2,3,4,6-tetramethylglucose, 2,3,6-trimethylglucose, 2,3,4-trimethylglucose, 3,4,6-trimethylglucose and 2,4,6-trimethylglucose.

RESULTS AND DISCUSSION

Differential temperature detection of sugars

In preliminary work in this laboratory[2], detection was effected at one temperature setting and it was not possible to discriminate between different compounds. In this work, an important advance has been achieved by varying the temperature range. The results obtained show a characteristic temperature detection range for each of the sugars tested.

About 1 μmol of the compounds listed in Fig. 1 was spotted on a TLC plate and chromatographed using solvent system B. After chromatography, the plate was placed in the gas chromatograph oven as described under Experimental. This method was chosen because of the accuracy in setting the desired temperature. Further, the time required to achieve an increment of 10°C was about 30 sec with this equipment. These characteristics were important for establishing the UV detection temperatures for each compound tested. Fig. 1 shows that detection under visible light is also possible with this procedure and that there is a characteristic detection temperature for each type of sugar.

It must be noted that there is a temperature range of about 20°C in which the compounds tested can only be detected under UV light. This is important with regard to the eventual high recovery of the compounds, as will be shown below.

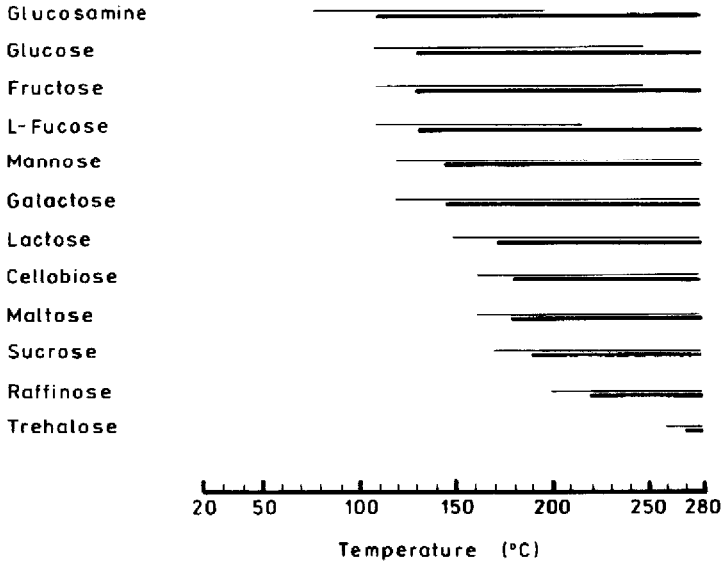

Fig. 1. Temperature dependence detection pattern of different sugars. Horizontal bars indicate detection temperature under UV (thin) or visible (thick) light. The compounds were subjected to TLC as described in the text. Detection was carried out by observing the plates after heating for 5 min. Temperature increments were made in 10°C steps.

According to their detection temperature, the sugars may be grouped into mono- and disaccharides. It appears that the disaccharides require a higher temperature for detection. The only trisaccharide tested, raffinose, possesses an even higher detection temperature than that of the disaccharides, with the exception of trehalose, which possesses the highest detection temperature of all the compounds tested. In the monosaccharide group, glucosamine had a very low detection temperature (60°C). However, if the amino group of glucosamine is blocked by an acetyl group the required temperature for detection is about 200°C (data not shown).

In addition, as shown in Fig. 1, fluorescence was lost for some compounds at high temperatures, probably owing to the quenching produced by the dark visible spot.

In order to determine whether the successive heating steps influence the detection temperature, the following experiment was carried out. The temperature was set directly at the UV detection values for each compound and maintained for 5 min. This was done for glucosamine (60°C), mannose (120°C), trehalose (260°C), raffinose (200°C) and sucrose (170°C). The results obtained were similar to those shown in Fig. 1. Therefore, stepwise heating does not influence the detection temperatures.

To determine whether the detection temperatures of a given compound may be influenced by the presence of other substances, the following experiment was carried out. A mixture composed of a substance developing at low temperature with another detectable at high temperature was spotted on a TLC plate and heated. The thermal treatment was carried out as indicated in Fig. 1. It was observed that the characteristic detection temperature for a given compound was not inluenced by the presence of another substance. This observation is restricted to the compound developing at the lower temperature. However, not all the possible interactions are ruled out by these experiments.

Equal amounts of the compounds of the following mixtures were used in these experiments: glucosamine–glucose, glucose–sucrose and sucrose–trehalose.

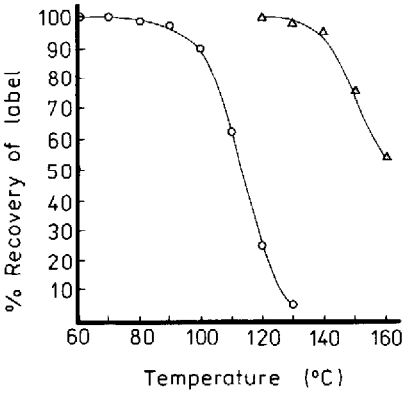

Fig. 2. Recovery of glucosamine and mannose after thermal–UV detection. [^{14}C]Mannose and [^{3}H]glucosamine (20,000 dpm, 1 μmol of each) were spotted on the plates and run in solvent system B. Each point corresponds to the radioactivity counted from the spots detected following the procedure indicated in Fig. 1. The recoveries are referred to the respective unheated samples. ○, Glucosamine; △, mannose.

Sensitivity for carbohydrates

In order to determine the minimal amount of sugar that could be detected by the thermal–UV method, different amounts of several compounds were chromatographed using solvent system B. It was found that 1 nmol of glucosamine, glucose and mannose could be detected under UV light after heating for 5 min at 200°C.

Recovery of [^{3}H]glucosamine and [^{14}C]mannose after thermal–UV detection

To determine the effect of the heating step on the recovery of [6-^{3}H]glucosamine and [U-^{14}C]mannose, about 20,000 dpm and 1 μmol of each were spotted on TLC plates. Ten spots for each substance were chromatographed using solvent system B, then the plate was heated at 60°C for 5 min. Subsequently, the glucosamine spot became visible under UV light; it was scraped off and counted for radioactivity. Thereafter, the plate was again heated to 70°C and kept for 5 min at that temperature. This step was repeated several times until 130°C was reached. In each step one spot corresponding to glucosamine was scraped off and counted. At about 120°C the mannose spots became detectable under UV light. A similar procedure to that described for glucosamine was carried out for mannose but starting from 120°C. The results are shown in Fig. 2. It can be seen that the recovery for both sugars was about 100% in the UV detection range. The recovery decreased as the temperature increased and the spots became detectable under visible light.

Detection of lipids

To extend the application of the thermal–UV method to lipids, the following compounds were chromatographed and the plates were heated at 200°C for 15 min. (a) standard lipids chromatographed in solvent system C (25 μg of each): β-sitosterol, geraniol, dolichol, squalene and cholesterol. (b) Brain lipids chromatographed in solvent system D: cerebrosides, ethanolamine phospholipid, sulphatide, lecithin, sphingomyelin and serine phospholipid. (c) Brain lipids chromatographed in solvent system E: free and sterified cholesterol, triglycerides, 1,2- and 1,3-diglycerides and free fatty acids.

All the compounds tested could be detected under either UV or visible light (data not shown).

Sensitivity for lipids

To examine the sensitivity of the method for lipids, cholesterol was spotted in different amounts, run using solvent system B and detected by heating at 200°C for 15 min. A 1-μg amount of cholesterol was easily detected under UV light (see Fig. 3). Similar results were observed with dolichol and oleic acid (data not shown).

The results obtained indicate that the thermal–UV detection method may be used with advantage over sulphuric acid-based methods.

The sensitivity of the method is very high in comparison with the methods in routine use. Further, it provides the advantage of having a very low background even after 12 h of heating at 200°C.

Detection of partially methylated glucose derivatives

Between 100 and 200 nmol of partially methylated glucose derivatives were run on TLC plates using solvent system A, then the plates were heated at 140°C for 15

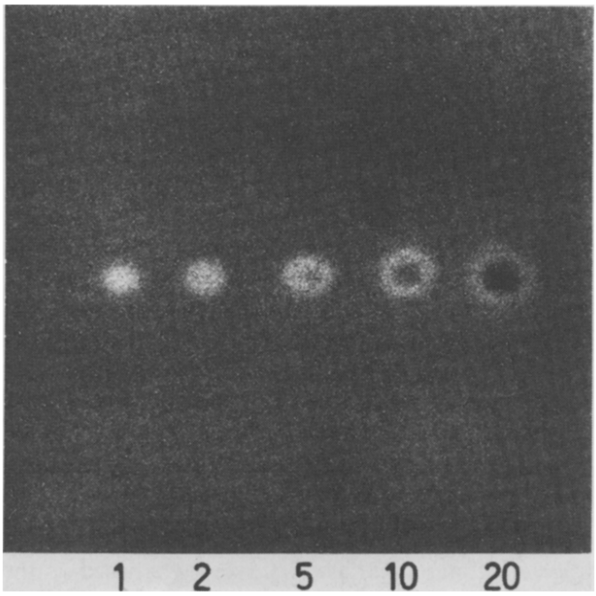

Fig. 3. Detection of cholesterol by TLC with the thermal–UV method. From 1 to 20 μg of cholesterol were spotted on the TLC plates, run in solvent E and heated for 15 min at 200°C. Numbers indicate amount of cholesterol (μg).

min. The compounds on the plates could then be observed using visible or UV light as shown in Fig. 4. However, if the heating was carried out for 3–5 min, the spots were detected only with UV light. Partially methylated derivatives other than glucose (such as mannose, rhamnose and galactose) gave similar results (data not shown).

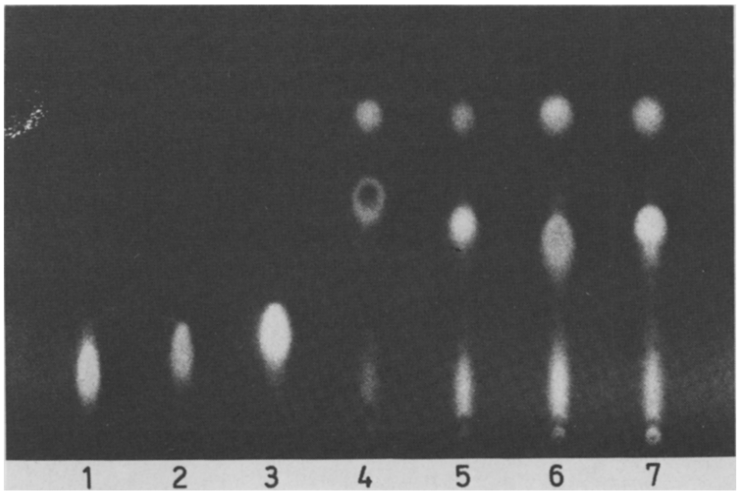

Fig. 4. Thermal–UV detection of partially methylated glucose derivatives subjected to TLC. Lane 1, 4,6-dimethylglucose; lane 2, 3,4-dimethylglucose; lane 3, 2,4-dimethylglucose; lane 4, 2,3,6-trimethylglucose; lane 5, 2,4,6-trimethylglucose; lane 6, 3,4,6-trimethylglucose; lane 7, 2,3,4-trimethylglucose. Lanes 4–7 contained also the tetramethylated derivative.

Detection of these compounds with sulphuric acid-based methods leads to the destruction of the samples[8]. In contrast, the thermal–UV detection method described here offers the advantage of a good recovery of the sample, as will be shown below.

Recovery of the samples

To evaluate sample recovery after detection, the following experiment was performed. About 200 nmol (4000 cpm of ^{14}C) of 2,3,4,6-tetramethylglucose was run in solvent system A. The plate was heated at 180°C for 3 min and only one spot was detected when observed under UV light. The plate was scanned for radioactivity (Fig. 5A) to confirm that the position of the radioactive peak and the UV-detected spot coincided. To determine whether the UV-detected compound retained its chromatographic properties it was first eluted with methanol–water (1:1) and subsequently spotted on a TLC plate, run as before and detected by scanning the radioactivity. As shown in Fig. 5B, the sample eluted from the heated plate (Fig. 5A) has an R_F value identical with that in the previous run. Another sample of the [^{14}C]tetramethylglucose derivative was processed under similar conditions with the exception of the heating step (control; data not shown). The recovery of the heated sample was 77% of that of the control.

It is worth noting that only a single radioactive peak was found after heating and rechromatography, which suggests that the product generated during heating remained bound to the silica gel and was not eluted.

Characteristic colour development after thermal–UV treatment

Before or after chromatography the compounds submitted to the thermal treatment develop characteristic colours under UV light (366 nm), *e.g.*, red for 2,3,6-trimethylglucose, white for 2,3,4-trimethylglucose, yellow for 2,4,6-trimethylglucose, blue for glucosamine, light yellow for N-acetylglucosamine and dark yellow for glucose. This phenomenon has been consistently observed when similar amounts of the above substances were heated in the temperature range which corresponds to UV

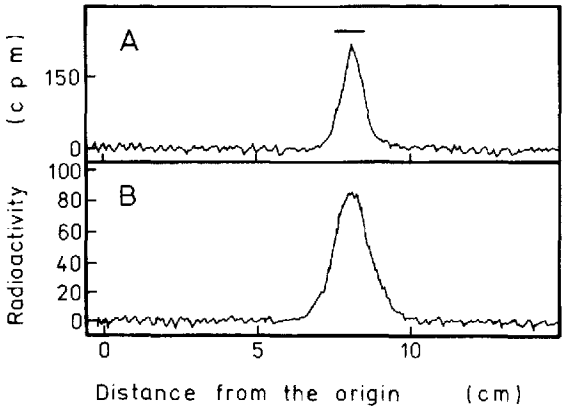

Fig. 5. Recovery of tetramethylglucose after thermal–UV detection. (A) Radiochromatogram of 4000 cpm and 250 nmol of 2,3,4,6-tetramethylglucose after TLC using solvent system A. The bar indicates the location of the UV-detected spot after heating. (B) Radiochromatogram of the material eluted from A and subjected to TLC under similar conditions.

light detection only (see above and Fig. 1). Longer periods of heating or higher temperatures lead to a shift in colour formation in most of the compounds.

It should be noted that the substitution at C-2 of glucose by an amino group shifts the colour from dark yellow to blue. Further, when the amino group of glucosamine is blocked by an acetyl group the colour changes to light yellow. It should be remembered that these compounds develop at different temperatures (*e.g.*, 110°C for glucose, 60°C for glucosamine and 180°C for N-acetylglucosamine).

The influence of structure on colour development is also illustrated by the trimethylglucosides. A change in the position of the methyl group from C-4 to C-6 is associated with a change in colour from white to red. Moreover, a change in the methyl group from C-3 to C-4 shifts the colour from red to yellow. Spectroscopic studies in combination with the thermal treatment may prove to be very useful in characterizing such organic compounds.

In conclusion, the method described here offers the possibility of discriminating amongst substances according to their detection temperatures and colours.

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