On the relationship between amount of substance and spot size in thin-layer chromatography

PURDY AND TRUTER[1], and TRUTER[2] discussed the possible relationships between the amount of substance per spot and the resulting spot size in thin-layer chromatography. They quoted the experiences of STAHL et al.[3] showing that, for thin-layer chromatography on silica gel, log weight is not always proportional to spot area.

Out of 10 substances tested by PURDY AND TRUTER[1] on silica gel, the best linear fit was with the relationship between log weight and the square root of the area. The relation between log weight and area gave a less good fit with the actual results.

An "unexpected feature", however, was that some of the substances gave reasonably good linear fits both with the areas and with the square roots of the areas.

The present author studied the relationship between amount of substance and spot area for the common sugars and organic acids, as well as for some amino acids, whereupon some other "unexpected features" turned up.

Technical procedures

Cellulose, MN 300 was spread on 12 × 16 cm plates as described by NYBOM[4]. Solvents were, for acids and sugars: ethyl acetate–formic acid–water (3:1:1) and for the amino acids: isopropanol–formic acid–water (20:1:5). Spots were developed with: bromphenol blue (0.016 % BFB + 0.085 % NaAc added to the solvent before separation) for acids; benzidine (cf. LINSKENS[5]), for sugars; and isatin[6] and ninhydrin (0.2 % ninhydrin in 5 ml collidine + 20 ml acetic acid + 75 ml methanol) for amino acids. The spots were copied on millimetre tracing paper, and the areas were determined by counting the millimetre squares under weak magnification.

Results

It was found that different relationships existed for log weight as a function of the area, depending upon the thickness of the cellulose layer.

Figs. 1 and 2 show this for citric acid and sucrose, but the same general tendency characterized malic acid, as well as glucose and fructose to the same degree.

Slits, 200, 400, 600, 800 and 1,000 μ wide, were used with the Balsgård spreader[4]. Layers intermediate in thickness gave curves falling between those of both extremes.

As was evident from the figures, a thin layer gave a linear relationship between log weight and area, whereas a thick layer gave a line relating log weight and the square root of the area.

The 350 μ layers, generally used with cellulose powder, gave reasonably linear fits for the relationship log weight–area for all sugars and acids tested (cf. Fig. 7).

As can be seen from Figs. 1 and 2, the spots for corresponding amounts were considerably bigger on the thin layer than on the thick layer. Thus, the thinner layer is more sensitive and will reveal smaller amounts. On the other hand, 48 μg is too large a quantity to be properly separated on the thinner layer.

When studying the amount–spot size relationships for amino acids, something similar occurred, although this time the same layer thickness, 350 μ, was used in all cases, but the spots were revealed by two different methods, viz. with isatin and with ninhydrin.
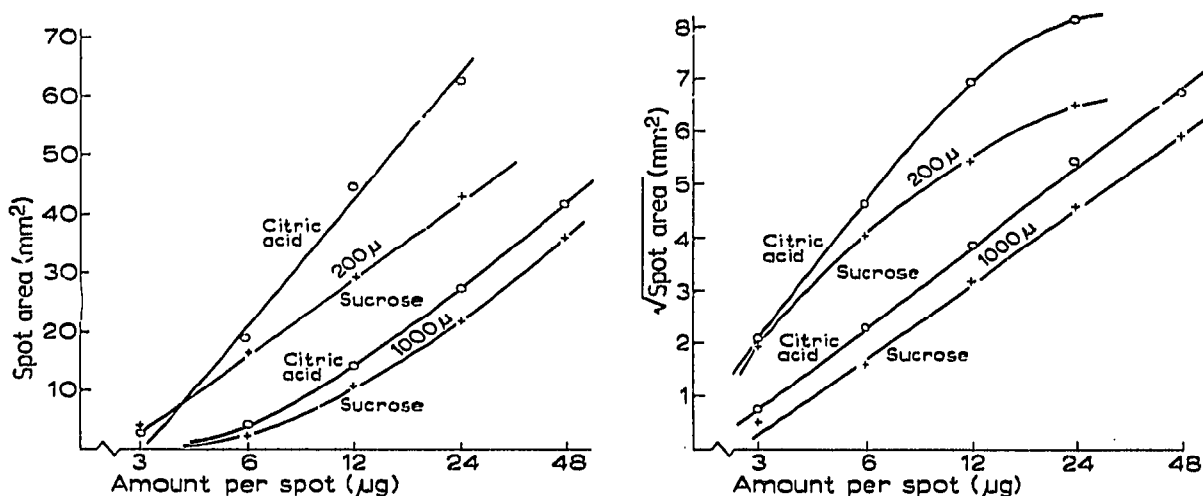
Fig. 1. The relationship between amount of substance per spot, on a logarithmic scale, and the resulting spot area. (O— O — O —) Citric acid; (+—+—+—) sucrose. Bent curves are obtained for the thick layer (1,000 μ) and straight lines for the thin layer (200 μ).

Fig. 2. The same material as in Fig. 1 with the square of the spot area along the ordinate. The thicker layer now gives straight lines, in contrast to the bent curves obtained for the thin layer.

As shown in Figs. 3 and 4, the isatin development gave a linear fit for the relationship log amount–log area, whereas ninhydrin, in Figs. 5 and 6, gave a straight line for the relationship log amount–area. Now, isatin is a less sensitive reagent than ninhydrin, spots containing corresponding amounts becoming considerably bigger with ninhydrin. Thus, one may say that isatin simulates a thicker layer, whereas ninhydrin behaves as if the layer was thinner—both with regard to spot size and with regard to the relationship between amount and spot size. Other amino acids behaved in the same way.

It should be mentioned that the relationship log weight–square root of the area is intermediate in curvature to log weight–area and log weight–log area. Another relationship that might work with very thick layers and insensitive reagents is the relationship weight–area (non-logarithmic scales).
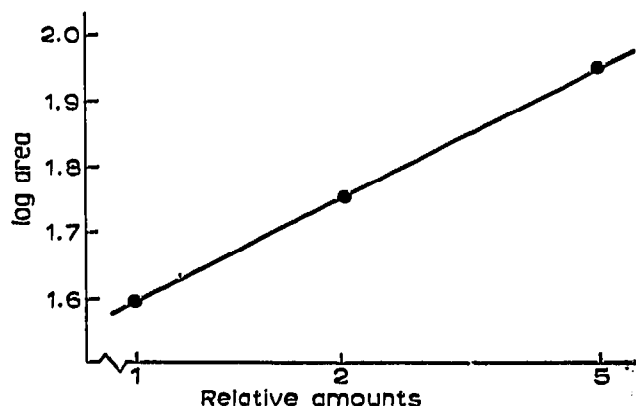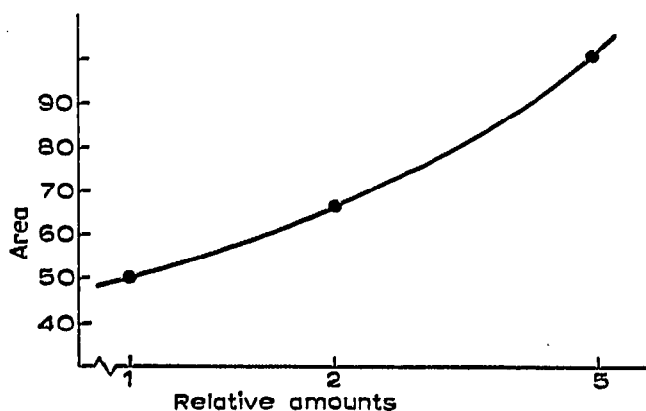
Figs. 3 and 4. Three different amounts of β-alanine on a logarithmic scale give a straight line relationship with log area when developed with isatin.
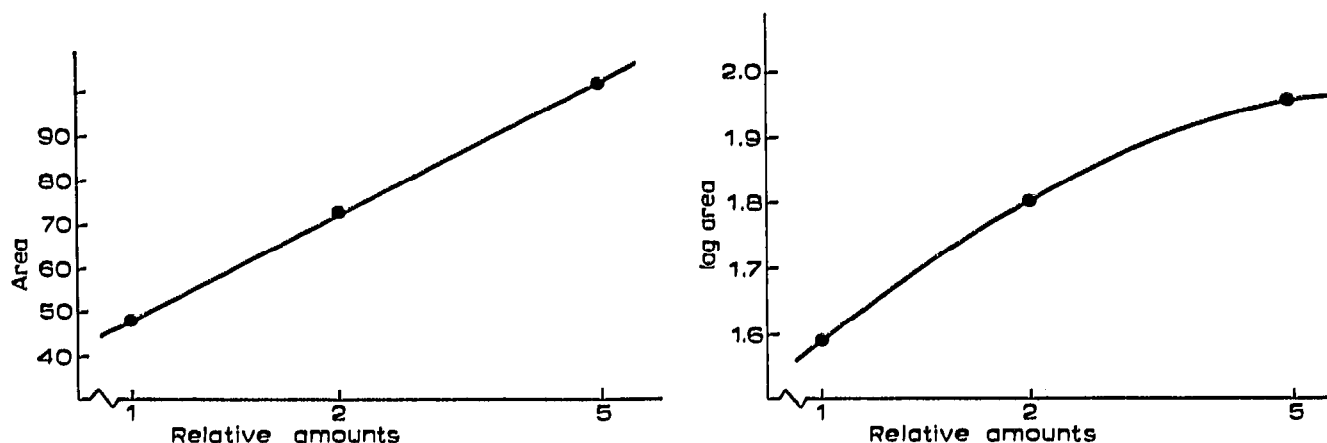
Figs. 5 and 6. After ninhydrin development, on the other hand, a straight line relationship is obtained between log amount and the areas of the spots.

Quantitative routine analysis

For the quantitative analysis, the spots are encircled as carefully as possible and traced on to semitransparent millimetre paper. Preparatory investigations have to be made in order to find out the actual relationship between amount per spot and spot size.

For the common sugars and organic acids, as shown in Fig. 7, straight lines are obtained if the logarithm of the weight of substance per spot is plotted against the surface area of the spots in a coordinate system.
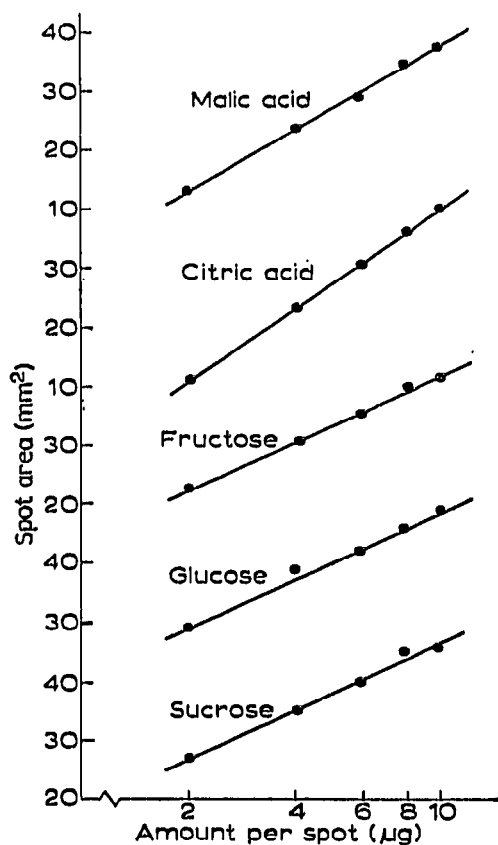

Fig. 7. Calibration curves for the common sugars and organic acids, showing the straight line relationships between log amount per spot and spot area.

Thus, the area $A = m \cdot \log w + c$, where m and c are constants, and w is the amount of substance in the spot. The area may be expressed in mm^2 and the weight in μg.

The constants m and c vary considerably from plate to plate, especially c; m being more constant. In one experiment with glucose, with six different plates, m varied between 41 and 56, with an average at *ca.* 50. The constant c varied between 4 and 21, with an average at *ca.* 13. This interplate variation with regard to m corresponds to errors of ± 25 %, whereas the variation in c gives errors of ± 40 %.

These constants, therefore, have to be determined separately for each plate, and at least two standard spots containing different known amounts are necessary per plate.

If the areas of these standard spots are determined for a TLC plate, the amounts in the unknown spots may be calculated according to the following formula:

$$\log w_x = \log w_{ds} + \log d \frac{A_x - A_{ds}}{A_s - A_{ds}},$$

where:

w_x = amount of substance in the spot from an unknown sample,

w_{ds} = amount of substance in the smaller diluted standard spot,

d = the relation (dilution factor) between the two standard spots,

A_x = surface of the unknown spot,

A_{ds} = surface of the smaller standard spot,

A_s = surface of the larger standard spot.

This arithmetic method is a modification of the procedures described by TRUTER[2].

It is often convenient to use standard spots containing 2 and 6 μg, respectively, of the substance in question. The dilution factor d will then be 3. The above formula then reduces to:

$$\log w_x = 0.778 \frac{A_x - A_{2s}}{A_{6s} - A_{2s}},$$

where $2s$ and $6s$ denote the spots having 2 and 6 μg of standard substance, respectively. To reduce the errors, which will be of the order of magnitude of 10–20 % for each determination, one should have two of each of the standard spots (2 and 6 μg) per plate. There will then be room enough for 8 spots from the samples to be analysed.

If it should turn out during the preparatory work that the relationship between log weight and area does not follow a straight line, one will have to try substituting areas with the square roots of the areas or with the logarithms of the areas. After such proper modifications, the above formulae can again be used in these cases.

Summing up, it is concluded that there does not appear to be any true, or *a priori* expected, theoretical relationship between amount per spot and spot size in thin-layer chromatography. The relationship will vary from substance to substance, depending on the chemical properties, and it will vary with the thickness of the layer as well as with the method used for revealing the spots. It will probably also depend on the absolute amount of substance in the spots, varying from one concentration range to another (*cf.* RANDERATH[7]).

However, for a fixed set of conditions, the determination of spot size lends itself fairly well for an, at least approximate, quantitative determination of the contents of the spots.

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1 S. J. PURDY AND E. V. TRUTER, *Analyst*, 87 (1962) 802.
2 E. V. TRUTER, *Thin Film Chromatography*, Cleaver-Hume, London, 1963.
3 E. STAHL, *Dünnschicht-Chromatographie*, Springer, Berlin, 1962; *Thin-layer Chromatography*, Springer, Berlin and Academic Press, New York, 1963.
4 N. NYBOM, *Balsgård Fruit Breeding Symposium, 1967*, p. 44.
5 H. F. LINSKENS, *Papierchromatographie in der Botanik*, 2nd Ed., Springer, Berlin, 1959, p. 94.
6 N. NYBOM, *Physiol. Plantarum*, 17 (1964) 434.
7 K. RANDERATH, *Thin-Layer Chromatography*, 2nd Ed., Academic Press, London, 1966, p. 71.

A thin-layer chromatographic method for distinguishing between natural rubber and synthetic polyisoprene

With the advent of increasing production of synthetic polyisoprene and subsequent use in commercial vulcanizates, there was an obvious need for a simple, reliable method for distinguishing between natural rubber and synthetic polyisoprene, to be used in a programme of research undertaken by the Association.

The present analytical methods, *e.g.*, the Weber test, infra-red spectroscopy etc., will only identify the polymers as *cis*-polyisoprene and will not differentiate between natural rubber and synthetic polyisoprene. Several methods have been published[1,2] which depend on the identification of a minor constituent of natural rubber not found in synthetic polyisoprene. The disadvantages of these methods are that they are time-consuming and present some uncertainty when dealing with rubber of unknown origin.

Natural rubber contains about 1 % w/w of extractable lipid materials[3,4] consisting of phospholipids, sterols, tocopherols, tocotrienols, carotenes, squalene etc., whereas synthetic polyisoprene will only contain ingredients added during manufacture. A new approach to this problem is based on examination of a solvent extract of the rubber by the technique of thin-layer chromatography, in order to characterize and distinguish between the lipid components in natural rubber and the additives in synthetic polyisoprene.

Experimental

Five grams (± 0.01 g) of the rubber sample (raw, gum or vulcanized: thinly sheeted on a mill) were extracted for 12 h with Analar acetone under reflux. After removal of the acetone by distillation the extract was dried at 105° for 10 min, dissolved in Analar carbon tetrachloride and diluted to 5 ml in a graduated flask. Silica gel (50 g) (Kieselgel G nach STAHL; neutral grade) was used as the substrate,