DETERMINATION OF KETO ACID DINITROPHENYLHYDRAZONES BY A GENERALLY APPLICABLE QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC METHOD BASED ON PHOTOMETRIC MEASUREMENT OF SPOT AREAS

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Quantitative thin-layer chromatography is most often based upon either elution of the compounds contained in the spots, direct photometry or fluorometry of the thin-layer plates or measurement of the spot areas[1-3]. Elution of the compounds in the spots is undoubtedly the most versatile and straightforward method. It is, however, tedious and the sensitivity is rather low because of the dilution introduced in the elution step and the possible interference from impurities in the supporting medium. The direct scanning procedures for thin-layer plates have recently been evaluated critically by KLAUS[4]. It appears that direct fluorimetry is the method which is most likely to give correct results, although the use of an optical system like the one published by WIEME[5] might overcome some of the difficulties with direct spectrophotometry. Direct scanning procedures have been developed very succesfully in connection with paper chromatography and electrophoresis[6] and some of these techniques might be valuable for thin-layer chromatography as well.

FISHER, PARSONS AND MORRISON[7] observed with paper chromatography that the spot areas are linearly related to the logarithm of the amount of substance present in the spots. A theoretical derivation based upon the laws of diffusion was given by BRIMLEY[8] (but see FISHER, PARSONS AND HOLMES[9]). The field was later reviewed by GIDDINGS AND KELLER[10] who extended the theoretical treatment. PURDY AND TRUTER[11] suggested, on the basis of results obtained by other investigators, that in the case of thin-layer chromatography a linear relation between the square root of the spot area and the logarithm of the content is most correct.

When measuring the spot area, the observer has to decide where the boundaries of the spot shall be placed. FISHER, PARSONS AND MORRISON[7] found that a high-contrast photoprint of the chromatogram was easier to measure than the chromatogram itself. Photographic copies of thin-layer chromatograms have been used for this purpose and for documentation of the chromatogram as well[1-3]. HEFENDEHL[12] prepared photoprints of thin-layer plates made transparent with paraffin and measured the spot areas by photometric measurements of these photoprints.

The reliability of the spot area method is, however, seriously affected by a problem which has been overlooked in most investigations. The spot areas of the analyses have to be compared with spot areas of standards of the same compounds and it is therefore necessary to assume that the distribution of substance within the

individual spot is identical in the standard and the analysis. If this is not the case, the spot area method will lead to erronous results. The extracts might very well contain substances which will change the concentration distribution in the spot to be measured. The use of internal standards is not always sufficient to reveal such interference.

The present paper describes an extension of the method of HEFENDEHL[12] based upon photometric scanning of photoprints of thin-layer plates. By scanning two or more photoprints obtained by different exposure of the same plate with both standards and samples, it can be checked that the sample spots and the standard spots have identical or nearly identical concentration distribution. The present method has been worked out for 2,4-dinitrophenylhydrazones of keto acids* (especially oxaloacetate) but might easily be applied to other compounds as well. It has been used successfully for polar and non-polar lipids developed with 50 % sulfuric acid and then heated (unpublished results). The modification of a commercial photometer for integral scanning of chromatograms and photoprints is described.

EXPERIMENTAL

Chromatography

Silica gel plates (20 × 20 cm) were prepared with the equipment obtained from Desaga, Heidelberg. 30 g kieselgel H nach Stahl (Merck, Darmstadt) was shaken vigorously with 70 ml water and spread in a layer about 0.2 mm thick. It was essential that the wet plates were left to dry at room temperature. If heat or forced air was used to dry the plates, very fragile layers were obtained. The silica gel contained impurities which absorbed ultraviolet light and which chromatographed in the medium with an R_F value of 0.7 to 1.0. This material could not be removed completely by development of the plates with various alkaline solvents before the chromatography.

The standard solutions of the K-DNPH were made up in 1 N ammonia and applied at the start as spots with an automatically driven microsyringe (usually 10 μl, catalogue No. 701 N, Hamilton Company Inc., Calif.). The mechanical device used to drive the syringe was an asynchronous motor coupled to a variable gear system and a threaded spindle. The thin-layer plates were pressed lightly against the end of the needle, which was ground in a right angle. The fluid was evaporated with a stream of air at room temperature. This set-up proved to be very useful and produced uniform start spots. 10 μl aqueous solution could be applied in about 15 min as a spot 3 mm in diameter. The rate of delivery was increased if volatile solvents or hot air ventilation was used because too dense start spots appeared to be unfavourable for the chromatographic separation.

The chromatograms were developed ascending in a saturated atmosphere with methanol–*n*-butanol–25 % ammonia (100 + 40 + 8 vol.). With some batches of silica gel the content of ammonia had to be lowered to 6 vol. in order to obtain satisfactory separation of OAA-DNPH and KG-DNPH. The start spots were placed about 2 cm from the end of the plate and 15 to 20 mm from each other. The front was allowed to reach 120 mm above the start spots. This would take about 45 min.

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* Abbreviations: K-DNPH = keto acid 2,4-dinitrophenylhydrazone. OAA-DNPH = oxaloacetate 2,4-dinitrophenylhydrazone. KG-DNPH = α-ketoglutarate 2,4-dinitrophenylhydrazone. Py-DNPH = pyruvate 2,4-dinitrophenylhydrazone.

Preparation of photoprints

The developed and dried plates were placed with the silica gel layer directly on the sensitive surface of the contact paper (Kodagraph contact paper, thin C 13). A 2 cm wide strip of metal sheet was placed along the lower end of the plate to shade this area of the photographic paper. The paper was exposed with a UVL-21 (Ultra-Violet Products Inc., Calif.) which was placed about 1.5 m above the paper to ensure uniform illumination. The contact paper was developed with Kodak D 163 developer for 2 min (continuous agitation) and the fixed and washed paper was dried in an electric contact print drier with the back of the paper against the polished metal plate.

The silica gel plates could easily be handled without damage and the contact between the layer and the sensitive surface of the contact paper was sufficiently good to eliminate most of the scattering. The use of paraffin for making the plates transparent[12] was thereby avoided. Reflex copies of the plates were of much lower quality than the ordinary contact copies.

The scanning procedure

The photoprints were cut in strips which had one side parallel to the centre line of the spots in a single lane (*cf.* Fig. 3). The strips were scanned in an Eppendorf photometer (Netheler & Hinz, GmbH, Hamburg) with the set-up shown in Fig. 1*.
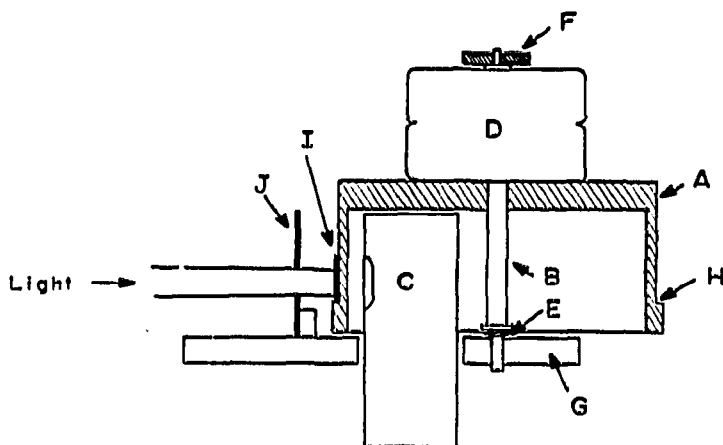

Fig. 1. The scanning set-up. Perspex cylinder (A) mounted on a metal shaft (B) could be rotated around the photocell (C) by the synchronous motor with gear (D). The motor was mounted on the cylinder, the position of which was secured by a spring (E) between the shaft and the base plate (G) and by the adjustable clamp (F) for the motor gear shaft. The cylinder had a small lip (H) and the strip of the photoprint (I) was held stretched over the polished surface above this lip (exposed side towards light source) by a piece of tape on each end. The light stop (J) was 0.8 × 10 mm.

Fig. 2 shows the electronic arrangement used for recording the transmission and the time integral of the transmission. This arrangement might be adapted to other photometers as well and other components than those indicated might be equally suitable for the purpose. With the present components, the full scale deflection of the recorders corresponded approximately to half the saturation value of the amplifiers. A peak would thereby be integrated properly even if the transmission recorder went a little off scale.

* It is probable that the equipment for scanning of electrophoresis strips (Pherogram-auswerter 2600) supplied for the Eppendorf photometer could be used for this purpose.

The scanning of a strip was performed by first adjusting the full scale control of the Eppendorf photometer to give transmission = 100 % with the white end of the strip in the light beam. When the clamp (Fig. 1, F) was tightened, the motor would turn the perspex cylinder. The strips were mounted on the perspex cylinder in such a way that the side parallel with the centre line of the spots rested on the lip (Fig. 1, H) and the spots were thereby passed through the centre of the light aperture. The strips were scanned at 546 mμ with a rate of about 1 mm/sec. The instrument was used in a shaded room and covered with black cloth during the scannings.
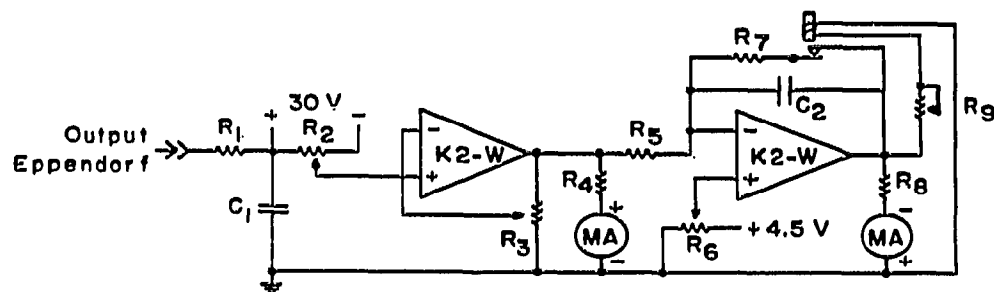

Fig. 2. The electronic arrangement for recording the transmission (100 % equals about 28 V) and its integral. The triangles indicate operational amplifiers (K 2-W, George A. Philbrick Res. Inc., Mass.) and the circles (MA) galvanometric recorders (1 mA recti/riter equipped with pen exciter system, Texas Instrument Inc., Texas). R_1, 1 MΩ. R_2, 20 kΩ 10-turn potentiometer (calibrated zero reset for transmission amplifier). R_3, 100 kΩ 10-turn potentiometer (calibrated gain control for transmission amplifier). R_4 and R_8, 25 kΩ (damping resistors for the recorders). R_5, 10 MΩ. R_6, 20 kΩ 10-turn potentiometer (zero control for integrating amplifier). R_7, 10 Ω. R_9, 250 kΩ potentiometer. C_1, 0.1 μF. C_2, 0.5 μF (mylar or equivalent). Some additional switches which facilitated the calibration are not shown. Power sources (not shown): Filaments, unstabilized alternating current; $\pm$ 300 V, a VR-tube stabilized supply (e.g. ref. 13); potentiometers, batteries.

Materials

The K-DNPH's were prepared from the keto acids (analytical grades) by a standard method[14]. The precipitated K-DNPH was recrystallized twice from *n*-butanol and showed only one spot when chromatographed with the present system. The standard solutions in 1 N ammonia were kept frozen at —15°.

All photographic materials were obtained from Kodak Ltd., London. Other chemicals were analytical grade.

RESULTS

Scanning and chromatography

Fig. 3 shows a strip of a photoprint and the recordings of the transmission (with unexposed paper as reference) and its integral. A mixture of OAA-DNPH (R_F 0.30), KG-DNPH (R_F 0.55) and Py-DNPH (R_F 0.69) was chromatographed. The strip was photographed in such a way that the abscissa of the recordings fit with the length coordinate of the strip.

The KG-DNPH spot (middle spot) had a very odd shape because this compound was chromatographed with R_F = 1.0 with respect to a secondary front which was unavoidable with solvents containing ammonia. The identification of the spot as KG-DNPH was therefore limited to the standards and could not be used uncritically for extracts of biological materials. The quantitative measurement of this spot was

also somewhat difficult, since its width might exceed the height of the scanning aperture.

The alkaline solutions of the K-DNPH isolated as described under materials never gave rise to formation of multiple spots of the K-DNPH (review, see NEISH[15]) in accordance with the finding of CAVALINI AND FRONTALI[16]. Apparently only one of the isomeric forms of each K-DNPH[17] was precipitated from the acid solution and no isomerization occurred during storage in ammonia and during chromatography.
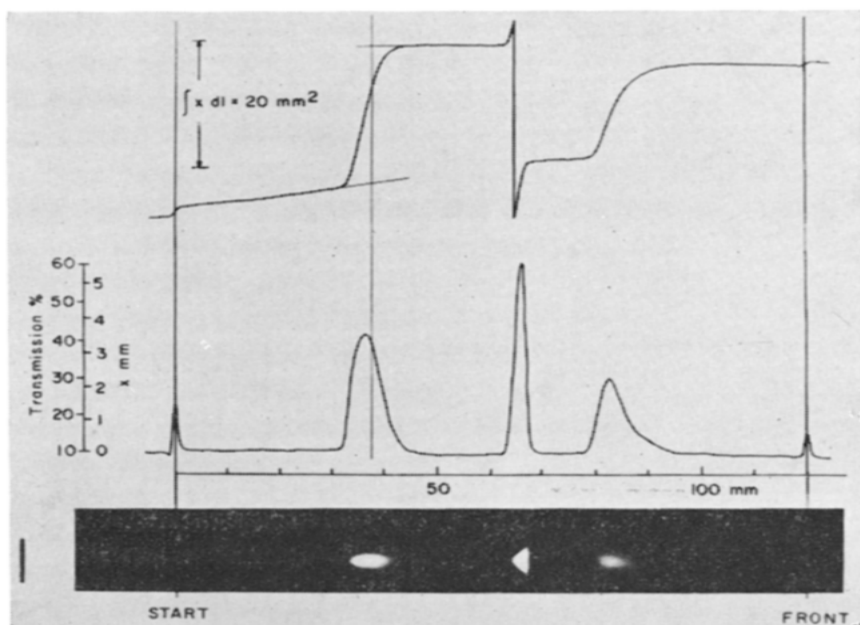

Fig. 3. Scanning of a strip of a photoprint. The strip is copied below the records. The spots are from left to right OAA-DNPH (4.7 nmoles), KG-DNPH (4.3 nmoles) and Py-DNPH (5.5 nmoles). The black bar at the left end of the strip indicates the position of the light aperture used for the scanning. The start and the front were marked with pinholes (see transmission record). For further explanation, see Section Scanning and chromatography. (Expt. 0004G).

This assumption was supported by a recent investigation of the isomerization of dinitrophenylhydrazones of aliphatic aldehydes and ketones[18]. There was no indication of decarboxylation of OAA-DNPH unless the undeveloped plate was kept for several hours after the spots had been applied at the start. BACHELARD[19] recently described the formation of two spots of OAA-DNPH on cellulose plates developed with *n*-butanol–ethanol–0.5 N ammonia (7 + 1 + 2 vol.) the solution of OAA-DNPH gave only one spot with the present system.

The concentration of substance in an ideal spot is highest in the centre of the spot and decreases gradually towards the edge, thus forming a bell-shaped distribution[8,10]. This implies that a high-contrast photoprint of the spot will show an unexposed centre area surrounded by an edge with gradually decreasing transmission, and that the area of the unexposed centre will be controlled by the exposure used for obtaining the photoprint in a way determined by the distribution of substance within the spot on the chromatogram.

The total transmission of a spot on the photoprint will be determined by the area of the unexposed centre and the shape and size of the edge zone. In order to

simplify this rather involved relationship, the transmission of the strip recorded with the scanning set-up was expressed as:

$$T = \frac{100 - T_b}{a} x + T_b$$

T = transmission in % (unexposed paper reference).
T_b = transmission of the background in %.
a = length of the light aperture used for the scanning (10 mm).
x = equivalent spot width.

The equivalent width of a spot was defined as the width which, provided the spot had a sharp edge, would give the same transmission as the actual spot.

The integral record could be expressed as:

$$k \frac{100 - T_b}{a} \int_0^l x \mathrm{d}l + k T_b \int_0^l \mathrm{d}l$$

l = length coordinate of the strip.
k = proportionality factor introduced to correct for the gain of the integrating amplifier and the speed of the synchronous motor.

The term $\int x \mathrm{d}l$ was defined in analogy with the equivalent spot width and called the equivalent spot area.

The peak area was calculated from the records as shown in Fig. 3 for OAA-DNPH (left spot). The integral record was placed over the transmission record and a line passing through the middle (not the maximum) of the peak was drawn. The area of the peak could then be calculated from the length of the intercept between the tangents to the integral curve and the first line. In this way a simple but rather reliable correction for changes in the background of the photoprint was obtained. If the integrating amplifier was zeroed by the relay as shown in the KG-DNPH peak (Fig. 3), the area was calculated in the usual way and the value added to the signal which would activate the relay. This signal would be constant for all scannings provided the zero of the integrating amplifier was adjusted appropriately by the potentiometer R_6 (Fig. 2) and the position of the potentiometer R_0 was unchanged.

Standard curves

Fig. 4 shows the standard curves (equivalent spot area *versus* amount of OAA-DNPH) obtained from six different photoprints of the same thin-layer plate. Increased exposure caused the curves to change shape but with a certain exposure (T_b about 30%), the standard curve was almost linear. In practice, however, it was always necessary to apply standards as well as samples to the same plate because it was impossible to relate the scannings of one plate with those of another. For routine applications the integrals, measured in arbitrary units, were compared and the equivalent spot areas were not calculated. The gain of the transmission amplifier was adjusted to give an appropriate peak size for all background transmissions. In this way the small values of the equivalent spot areas were scanned with the same relative sensitivity as the large values.

The maximal equivalent spot widths (*i.e.* the peak heights from the transmission

records) were found to indicate the content of the spots as well. The spot width was more sensitive to distortion and less favourable as a basis for quantitation than the spot areas.
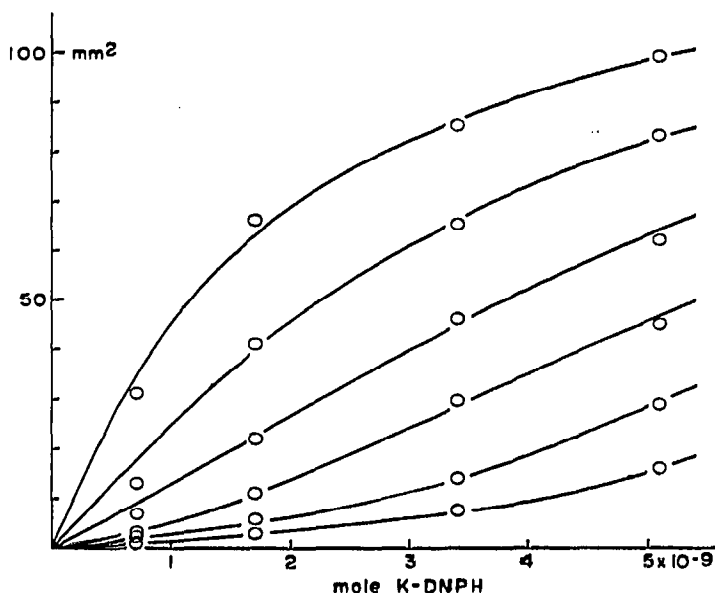

Fig. 4. Standard curves for OAA-DNPH. Ordinate is equivalent spot area. Each curve represents scannings of a photoprint. The background transmissions of the photoprints were from top to bottom: 68 %, 46 %, 31 %, 21 %, 15 % and 12 %, respectively (cf. Table II). (Expt. H 0109).

The standard curves shown were obtained with OAA-DNPH, but identical curves were given by KG-DNPH and Py-DNPH. Variation of the exposure would, however, affect the equivalent spot area of KG-DNPH much less than for OAA-DNPH and Py-DNPH. In some cases the standard curves for KG-DNPH would not pass through the origin because the secondary front would leave a small amount of ultraviolet absorbing material in the plate. The determination of pyruvate with the present solvent system was hampered by a rather poor separation and by the impurities in the silica gel.

Accidental errors and sensitivity of the method

The accidental errors of the method might primarily arise from instrumental errors, variation in the transmission of the photographic paper and the thin-layer plate, and variation of the spots on the plate. With the present instrument, the errors of the scanning *per se* were negligible, while the variation in the transmission of the photographic paper appeared to be limiting for the sensitivity of the method. The variation in transmission of the thin-layer plates was not critical, when the plates with abruptly changing thickness (waves) or scratches on the glass surface were discarded.

The standard error on a single determination (Table I) appeared to be of the order of ± 10 % (8–9 spots) of the amount of OAA-DNPH. KG-DNPH was scanned with about the same accuracy. The experiment with a high amount of OAA-DNPH (H 0158) showed a somewhat higher error than the rest. In other experiments with the same amount and number of spots an error around 7 % was found. This dis-

TABLE I

STANDARD ERRORS ON A SINGLE DETERMINATION OF OAA-DNPH

The same volume of standard solution (5–7 µl) was applied in all start spots on each plate. The errors on the amounts were obtained by dividing the errors on the equivalent spot areas by the slope of a standard curve which from the background transmission and equivalent spot area was judged to apply to the scanning.

Expt. No.	Background transmission (%)	Max. equivalent spot width (mm)	Equivalent spot area (mm²)	Amount of OAA-DNPH (nmoles)	Standard error in % of amount
H 0115 8 spots	62	3.1 ± 0.30	23 ± 2.1	0.54 ± 0.053	10
	31	0.93 ± 0.12	7.4 ± 0.71	0.54 ± 0.051	9
	18	0.40 ± 0.073	2.8 ± 0.47	0.54 ± 0.094	17
H 0160 9 spots	61	4.9 ± 0.37	52 ± 2.9	1.5 ± 0.13	9
	34	3.2 ± 0.21	29 ± 2.4	1.5 ± 0.13	9
	15	1.4 ± 0.13	12 ± 1.2	1.5 ± 0.15	10
	7.4	0.58 ± 0.053	4.7 ± 0.35	1.5 ± 0.088	6
H 0158 9 spots	57	6.5 ± 0.53	81 ± 4.5	3.0 ± 0.41	14
	18	4.25 ± 0.087	37 ± 2.5	3.0 ± 0.21	7
	6.8	1.6 ± 0.24	11 ± 2.0	3.0 ± 0.40	13
	4.5	0.39 ± 0.056	2.6 ± 0.53	3.0 ± 0.35	12

crepancy was explained by the variation of the R_F value of the secondary front caused by insufficient saturation of the tank atmosphere. In the case of Expt. H 0158 this value varied from 0.48 to 0.56. The maximal spot widths and the equivalent spot areas appeared to be correlated to the distance moved by the secondary front, but the correlation was not simple and would depend, for instance, on the background transmission of the photoprint.

In general the principal source of error appeared to be the difference in behaviour of the spots as they moved up the plate. The error of the volume of solution applied in the start spots and the variation in area of the start spots produced by the mechanical device were of minor importance for the total error of the method. The variation in the transmission of the photographic paper was only critical with low amounts of K-DNPH. The scanning of Expt. H 0115, 18 % background transmission, might have been limited by this variation, which most likely was caused by inhomogeneities in the paper base. Scanning of reflected light instead of transmitted light might improve the accuracy of this step in the method. The very thin contact paper (Kodagraph contact paper, ultrathin C 12) showed the same lack of homogeneity as the present paper. Sheet film (Kodak CF 7) offered no essential improvement because the grains in the thin-layer were copied as a result of the larger useful exposure range given by this material.

The minimal amount of OAA-DNPH which could be seen on a photoprint was about 0.1 nmole, while direct observation of the plates in ultraviolet light would need at least 5 times this amount for detection of a spot. The presence of 0.2–0.4 nmoles of OAA-DNPH could be established from the records. KG-DNPH yielding a more dense spot was detected with a higher sensitivity.

Systematic errors

As mentioned above a serious systematic error of the spot area method is the

possible difference in the distribution of substance in the sample spots and in the standard spots. Fumarate present in the solution applied in the start spots was found to change the concentration distribution of OAA-DNPH because fumarate was chromatographed in front of OAA-DNPH (R_F fumarate = 110% of R_F OAA-DNPH). 20 nmoles of fumarate would cause a distortion of the OAA-DNPH spot which could be seen directly on the photoprint. Higher amounts of fumarate might block the longitudinal spreading of the OAA-DNPH spot completely, while smaller amounts in most cases would give a distortion of the OAA-DNPH spot which could scarcely be observed on the photoprints. The effect of a certain amount of fumarate appeared to be higher on chromatograms where the distance between the KG-DNPH and OAA-DNPH spots was small.

TABLE II

ERRORS IN COMPARING A SCANNING OF OAA-DNPH IN A SAMPLE CONTAINING FUMARATE (9 NMOLES) WITH A STANDARD CURVE WITHOUT FUMARATE IN PER CENT OF THE AMOUNT READ FROM THE STANDARD CURVE

Positive errors indicate that the amount determined with fumarate was too high, negative errors too low. For further description, see Section Systematic errors. (Expt. H. 0109).

Relative exposure	Background transmission (%)	nmoles OAA-DNPH read from standard curve without fumarate		
		1.0	3.0	5.0
1.00	68	—60 %	—60 %	—58 %
1.26	46	+30 %	0	—20 %
1.60	31	+30 %	+13 %	—14 %
2.00	21	+30 %	+17 %	+ 8 %
2.65	15	+40 %	+23 %	+10 %
3.40	12	+40 %	+23 %	+14 %
4.40	11	+40 %	+30 %	+16 %
6.60	10	+40 %	+30 %	+16 %

The data of Table II were calculated from two standard curves on the same plate, one without fumarate and one with 9 nmoles of fumarate present in the spots at the start. The table gives the error which would result from a comparison of a scanning of a spot where fumarate was present in the chromatogram with a standard curve without fumarate in per cent of the amount read from the standard curve (1, 3 and 5 nmoles). If, for instance, a spot on a photoprint with background transmission 68 % was found to contain 1.0 nmole OAA-DNPH, the true content of OAA-DNPH in this spot would be 1.6 nmole if 9 nmoles fumarate was present in the extract applied on the start.

As seen from Table II the effect of fumarate at low exposures was to make the spot look smaller and at higher exposures to make it look larger. With a certain amount of OAA-DNPH, fumarate would be without effect, but this amount was dependent on the exposure. The distribution of errors therefore seemed so complicated that internal standards would be of no use for correcting the results. It appeared that the determination of higher amounts was relatively less affected by fumarate.

With extracts of biological materials the problem was to detect a possible interference and to eliminate this by modifications of the extract procedure. The pre-

sence of lipids or small amounts of salts (*e.g.* from absorption of carbon dioxide in the ammonia) or too small start spots would in some cases change the concentration distribution. When the method was used for these extracts at least four photoprints were made of each plate with a relative exposure difference of about 2.5 times and the highest background transmission about 60%. Three or more of these were scanned and the content of the spots read from the standard curves. The results were generally accepted if the difference between the highest and the lowest value was less than 20 % of the mean. The standard error of a determination would be lowered somewhat by taking the mean of the scannings of the different photoprints. The error in Expt. H 0160 (Table I) calculated in this way was ± 7 % of the amount of OAA-DNPH.

DISCUSSION

The present method might be applied to the quantitative determination of any compound which can be separated by thin-layer chromatography and made visible on a photoprint of the plate. It is probable that a photograph of the thin-layer plate can be used for the quantitation as well. The method offers some essential advantages. The spot areas are measured objectively. It is non-destructive since the photoprint which in most cases has to be made for documentation is used for measurements. It might be advantageous with compounds which are too labile to permit effective elution from the plate. It has a high sensitivity and allows control of systematic errors.

The sensitivity of the method used for K-DNPH was about 0.2 to 0.4 nmoles. With other compounds or with different chromatographic systems giving denser spots an increased sensitivity of the method might be expected. Preliminary experiments with elution of the K-DNPH from the silica gel layer with 1 N NaOH resulted in a background extinction corresponding to about 2.5 nmoles. It is, however, likely that a more gentle elution would lower this background (*cf.* RANDERATH AND RANDERATH[20]). The very elegant scanning method for paper chromatograms developed by BUSH needs about 1.8 nmole of Py-DNPH for detection[6,21].

The high sensitivity makes the use of photoprints with low background transmission obligatory, and the possibility of systematic errors has to be eliminated by scanning of photoprints obtained with different exposures. This, together with the need to apply several standards (usually 4) to each plate makes the method rather time consuming (about 8 analyses a day). It is possible by sacrificing the reliability to cut down the numbers of standards on each plate and the numbers of differently exposed photoprints which have to be scanned. With high amounts of substance and less diffuse spots (*i.e.* with dark photoprints) this simplification might be justified (*cf.* ref. 12). The present results show that the recording of spot widths instead of spot areas in some cases might be sufficiently accurate. In fact only a little experience is needed to judge the content of a spot from direct observation of the photoprint. The accuracy of this judgment is, however, highly increased by knowing the scanning result. Planimetry was tried, but was less accurate with these small, diffuse spots.

SUMMARY

A quantitative thin-layer chromatographic method and its use for the determination of the dinitrophenylhydrazones of α-ketoglutaric and oxaloacetic acids is described. The method involves photometric scanning of photoprints of the thin-layer plates. By scanning several photoprints obtained with different exposures, it is possible to check that the concentration distribution of the standard spots and the sample spots are identical. The sources of errors of the method were investigated. The sensitivity of the method is at least 0.4 nmoles oxaloacetate dinitrophenylhydrazone and the standard error on a single determination about $\pm$ 10 %. The modification of a commercial photometer for integral scanning of chromatograms and photoprints is described in addition to a mechanical device for application of samples to the plates.

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