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RECOVERIES AND REPRODUCIBILITY IN THE THIN-LAYER CHROMATOGRAPHIC FLUORIMETRIC DETERMINATION OF FLAVINS BY THE ELUTION METHOD

PART VIII*. CHROMATOGRAPHY OF RIBOFLAVIN DECOMPOSITION PRODUCTS

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SUMMARY

For riboflavin and hydroxymethylflavin acetate, recoveries of the substances deposited on the layer (either with or without subsequent chromatography), eluted and subjected to fluorimetry increased with the amount of flavin applied. Variations in these recoveries did not exceed those of the reference determinations, which were liable only to volumetric and/or fluorimetric error. The recoveries decreased to a certain extent when the spot area was very large as well as with longer runs. Thin-layer chromatography on cellulose could not be used for hydroxymethylflavin acetate as it resulted in the degradation of this flavin ester.

INTRODUCTION

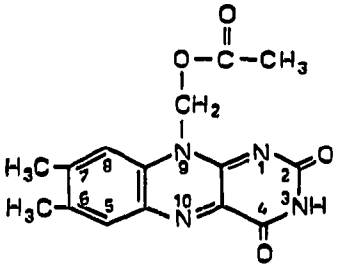
It is an obvious advantage of the *in situ* fluorimetric assay method compared with the elution method that it does not require the layer material to be removed from its support. If close or partially overlapping zones have to be cut apart on the basis of visual detection, the choice of the dividing line is more or less arbitrary. When the chromatogram is sectioned at regular intervals, part of the resolution is lost if the intervals are too great, and if they are small, the sensitivity is reduced and the manual workload is increased. Automated scraping such as that devised mainly for liquid scintillation counting of radioisotopes can relieve this workload[1–3]. Insufficiently reproducible results may represent a source of error. This study is therefore devoted to this aspect.

* For Part VII, see ref. 18.

In situ fluorimetry is, of course, subject to a number of other unavoidable errors, one of which is the interference of the sorbent if it either exhibits fluorescence or modifies that of the substance to be determined. The distribution of the substance across the layer and the size of its particles may affect the reproducibility as it depends on the detailed drying procedure adopted during spotting and subsequent to chromatography. Variable amounts of residual chromatographic solvent that remain after drying may also impair the reproducibility.

The accuracy of the *in situ* method has been reported in numerous papers[4–16] and a brief compilation of the accuracies (coefficients of variation) has been given by COURT[17]. In the present study, we have attempted to explore the dependence of the analytical yields and of their variation on the amount of substance applied, the nature of the sorbent and other variables. It could be expected, from the usual convex shape of the adsorption isotherm, that recoveries might be reduced in the instances of smaller amounts of sample, larger spot areas and longer runs.

For this purpose, we have chosen two flavins that differ in polarity, namely the classical substance of fluorimetric analysis, riboflavin*, which would be best eluted with aqueous solutions, and hydroxymethylflavin acetate** (see formula), which we had identified among the photolysis products of riboflavin and of formyl methylflavin***[18] and which could be eluted with an organic solvent.



We did not attempt parallel experiments with the *in situ* method, as it was likely to suffer from serious drawbacks in this particular instance and the comparison might not be valid. By simple inspection of the chromatogram under a 366-nm UV source, it can be observed that self-quenching occurs in the centres of compact thin-layer chromatographic (TLC) spots in amounts above 1 μg. In addition, the *in situ* fluorescence of the flavins depends critically on the extent to which the chromatographic solvent, which enhances it, has been removed.

EXPERIMENTAL

Layers (10 and 12.5 mg of sorbent per cm^2) were prepared by spreading 4 g of Kieselgel G (Merck) in 12 ml of water or 5 g of microgranular Cellulose CC 41 (Whatman) in 13 ml of butanol[19] on a 20 × 20 cm glass plate. After spreading,

* Riboflavin = 6,7-dimethyl-9-D-ribitylisoalloxazine. Merck product, chromatographically pure.

** Hydroxymethylflavin acetate (HMF acetate) = 6,7-dimethyl-9-acetoxymethylisoalloxazine. Prepared according to ref. 18; chromatographically pure except for a trace amount of lumichrome.

*** Formylmethylflavin = 6,7-dimethyl-9-formylmethylisoalloxazine.

the layers were left in the laboratory overnight and the silica gel layer was heated to 100° for 1 h. The plates were stored in a closed container.

The samples were diluted so as to apply 20 μl of the solutions from the same pipette in each series of experiments; the pipettes were not rinsed with pure solvent between the applications of identical samples.

Riboflavin was dissolved in 0.067 *M* phosphate buffer of pH 6.8 and HMF acetate was dissolved in chloroform–ethanol (1:1). Chromatograms were run with the organic layer from the *n*-butanol–acetic acid–water (4:1:5) mixture in an atmosphere saturated with the organic layer.* Most of the liquid was then removed with a stream of warm air and the chromatograms were left to dry at room temperature.

The spots were rapidly marked under a UV lamp (366 nm); identical areas were scraped off in each series and eluted for 10 min with 5 ml of the 0.067 *M* phosphate buffer of pH 6.81 for riboflavin and with 5 ml of ethanol for HMF acetate. The sorbent was removed from the suspension by centrifugation. The acidification of the buffer (down to pH 5–6) by the acetic acid that remains in the layer should not influence the fluorescence characteristics of riboflavin in the particular pH range[20,21].

In order to limit photolysis, chromatography was performed in complete darkness and all operations were carried out in subdued light. The results were corrected for background fluorescence (blank areas of the layer of identical size were extracted with the same solvent; the fluorescence reading did not differ from that of the solvent used for extraction). Fluorimetry was carried out with excitation at 470 nm for riboflavin and 475 nm for HMF acetate and an emission wavelength of 540 nm (uncorrected) using a DC/3000 spectrofluorimeter (Ciampolini, Florence, Italy).

The same amounts of the sample were transferred by pipette in the elution solvent in such a way that the tip of the pipette touched the inner surface of the test-tube above the surface of the liquid. Even this reference assay exhibited some scatter in the readings, possibly owing to the volumetric and/or fluorimetric error. Hence the 100 % values in our graphs, which serve as the basis for the calculation of the percentage recoveries from thin layers, will have coefficients of variation.

In experiments that involved spotting on layers without chromatography, the samples were applied in the usual way, wetted with the solvent (the mobile phase was allowed to ascend through the origin spot until the flavin spot just began to spread in the direction of flow), dried and eluted.

Most of the experimental groups comprised six samples, which were run simultaneously on one chromatogram. The recovery is considered to be the fluorimetric reading divided by the average reading from the corresponding group of reference assays. Recoveries defined in this way might be affected by any substance that is eluted from the layer which would modify the fluorescence.

Influence of the amount of substance applied

Table I shows the results of different experiments with silica gel layers. The

* The R_F values for riboflavin, HMF acetate and lumichrome were about 0.30, 0.45 and 0.60 on silica gel and 0.43, 0.60 and 0.58 on cellulose.

TABLE I

DEPENDENCE OF THE RECOVERIES OF RIBOFLAVIN AND HMF ACETATE ON THE AMOUNT OF SUBSTANCE SPOTTED ON THE SILICA GEL LAYER

Amount (μg)		*Riboflavin*					*HMF acetate*				
		Reference coeff. of variation	*Recovery*				*Reference coeff. of variation*	*Recovery*			
			Spotting		*TLC*			*Spotting*		*TLC*	
			Mean	*Coeff. of variation*	*Mean*	*Coeff. of variation*		*Mean*	*Coeff. of variation*	*Mean*	*Coeff. of variation*
0.05		6.7	—	—	82.1	2.6	5.4	—	—	79.5	2.6
		1.4	95.1	1.4	94.0	1.5	2.6	96.7	2.0	100.6	2.2
		2.0	94.7	3.9	82.5	2.2	1.5	85.1	1.7	86.0	1.4
		4.3	81.0	7.6	—	—					
	Mean	3.6	90.3	4.3	86.2	2.1	3.2	90.9	1.8	88.7	2.1
	S.D.	±2.4									
0.1		1.4	90.4	2.2	87.1	2.1	3.7	94.6	2.6	89.3	5.6
		2.6	—	—	85.7	3.9	2.5	—	—	84.9	2.4
		2.7	—	—	87.9	1.5	2.5	—	—	91.0	2.8
		2.3	85.2	0.6	85.2	1.1	5.9	—	—	89.0	3.3
							0.9	89.0	0.8	91.7	1.8
	Mean	2.3	87.8	1.4	86.5	2.2	3.1	91.8	1.7	89.2	3.2
	S.D.	±0.6			±1.2	±1.2	±3.4			±2.6	±1.5
0.25		1.5	92.2	0.8	88.7	1.6					
0.5		1.7	—	—	90.5	5.3	4.1	—	—	91.7	1.3
		2.7	—	—	89.0	4.7	3.0	100.5	1.4	97.3	2.4
		1.9	99.1	2.5	99.1	2.3	1.8	92.9	1.7	90.0	1.4
		4.4	95.4	3.4	86.3	1.5					
	Mean	2.7	97.2	3.0	91.2	3.4	3.0	96.7	1.6	93.0	1.7
	S.D.	±1.2			±5.5	±1.8					
1.0		3.4	—	—	89.9	2.0	4.2	92.6	2.2	91.5	1.6
		2.7	—	—	92.0	1.4	2.5	101.1	3.9	97.2	1.4
		1.5	91.0	3.1	93.0	3.2	2.3	97.1	1.7	95.4	1.5
		3.6	99.3	0.9	93.4	1.6					
	Mean	2.8	95.2	2.0	92.1	2.0	3.0	96.9	2.6	94.7	1.5
	S.D.	±0.9			±1.6	±0.8					
2.5		1.9	—	—	95.1	2.0	2.1	—	—	96.0	2.8
		1.6	97.9	3.2	89.5	0.6	1.6	99.0	3.1	86.3	4.2
		1.1	96.5	1.3	90.8	2.1	2.7	96.5	0.7	95.8	1.2
	Mean	1.5	97.2	2.2	91.8	1.6	2.1	97.7	1.9	92.7	2.7

S.D. values are the standard deviations calculated from the mean values* and from the corresponding coefficients of variation of the individual groups of six samples, provided that there were at least four such groups for the particular concentration level. The averaged group means and coefficients of variation were plotted in Fig. 1 for riboflavin and in Fig. 2 for HMF acetate.

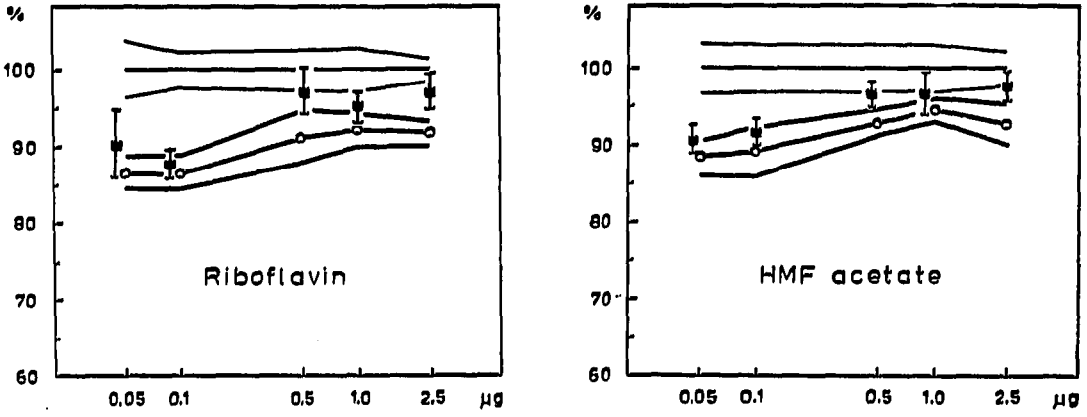


Fig. 1. Recoveries of riboflavin from silica gel thin layers expressed as a percentage of the mean reference determinations carried out simultaneously with the respective thin-layer experimental group. ○ = Average mean values for chromatographed samples; and ■ = average mean values for spotted unchromatographed samples. The average coefficients of variation are designated by the band width for reference and chromatographed samples and by vertical bars for spotted unchromatographed samples.

Fig. 2. Recoveries of hydroxymethylflavin acetate from silica gel thin layers. See legend of Fig. 1.

It can be seen that the recoveries tended to increase as the loads increased, for both the chromatographed and the spotted unchromatographed samples. Some of the results were subjected to statistical analysis by Student's t-test. The increase in the percentage recovery from 0.1 to 1.0 μg was significant ($p < 0.01$) for both the chromatographed and unchromatographed flavins.

The higher recoveries for the unchromatographed samples might have been due to some losses during the chromatographic run. The differences did not show, however, any regular dependence on concentration, which might suggest that the reduced recoveries for small samples were due to adsorption in the elution stage rather than to greater losses during chromatography. For the 2.5-μg samples, some losses during chromatography might have been due to limited solubility, as suggested by the tailing observed on the chromatograms.

Our recoveries are comparable with those for riboflavin obtained by paper chromatography (MASŁOWSKI[22], 80%). The reproducibility (coefficient of variation *ca.* 2%) is comparable with that of the *in situ* fluorimetric method described by JÄNCHEN[4] for harmane alkaloids (S.D. 1.9%) or Schoeffel Instruments Corp.[16] for aflatoxin (accuracy 2–4%).

The average variation of the results for chromatographed samples was mostly lower than that of the reference samples. Hence chromatography and elution

* These figures provide information about the day-to-day or plate-to-plate variations.

do not seem to add an additional accuracy-reducing factor to the volumetric[23] and/or fluorimetric error of the reference series.

For cellulose thin layers, HMF acetate could not be tested because the substance was degraded, mainly to lumichrome, when it came into contact with cellulose wetted with the solvent. Bleaching can be observed visually and recoveries may decrease to 25 %. For riboflavin, the recoveries were within the range 60–80 % and they also tended to increase with increasing amounts of sample (from 0.05 to 2.5 μg). The elution of unchromatographed spots gave variable results, some recoveries being lower than those of the corresponding chromatographed spots, but the differences between the chromatographed and undeveloped samples rarely exceeded 6 %.

Influence of the spot area

Table II shows the recoveries from chromatographed samples when the spot areas were deliberately reduced or increased. Although the differences between the pairs of groups were statistically significant, except for the riboflavin Experiment No. 2, the results did not reveal any regular dependence on spot size. When there was a very large surface area (Experiment No. 1, see Figs. 3 and 4), the difference was noticeable, the larger areas yielding lower recoveries.

TABLE II

COMPARISON OF RECOVERIES FROM COMPACT AND DIFFUSE SPOTS FOLLOWING TLC

Flavin	*Amount spotted (μg)*	*Expt. No.*	*Area after chromatography (cm^2)*		*Area ratio, diffuse/ compact*	*Recovery (%)*		
						Mean[a] ± S.D.		*Difference of the means, compact minus diffuse*
			Compact spot	*Diffuse spot*		*Compact spot*	*Diffuse spot*	
Riboflavin	1.0[b]	1	0.8	6.2	7.6	89.9 ± 1.8	75.6 ± 2.7	+ 14.3
	0.1[c]	2	0.7	2.5	3.6	85.7 ± 3.3	86.5 ± 3.0	− 0.8
		3	0.5	3.0	6.0	87.3 ± 2.4	80.4 ± 2.0	+ 6.9
HMF acetate	1.0[b]	1	1.9	8.2	4.3	86.6 ± 3.8	76.8 ± 1.5	+ 9.8
	0.1[c]	2	1.0	2.4	2.4	91.0 ± 2.5	88.2 ± 1.1	+ 2.8
		3	0.3	2.4	8.0	95.0 ± 1.8	98.5 ± 2.0	− 3.5
		4	0.4	2.3	5.8	86.1 ± 1.7	90.6 ± 1.4	− 4.5

[a] Results from each of the paired groups of TLC analyses under comparison were expressed as a percentage of the mean of the same group of reference readings.

[b] Each series consisted of eight samples from two plates. For the production of compact and diffuse spots, see the legend of Fig. 3.

[c] Each series consisted of six samples; both paired groups were developed simultaneously in the same tank allowing the solvent to ascend 4–6 cm. Compact and diffuse spots were produced by appropriate spotting on a minimal and maximal area, respectively.

The results suggest that spot size may influence the recoveries in the case of flavins, but that its influence is unlikely to invalidate the results if the spot areas do not differ to such an extent as in the present experiments.

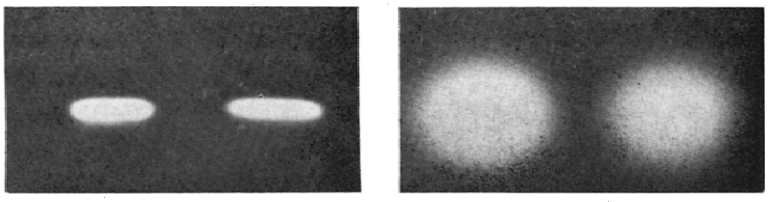


Fig 3. The appearance of HMF acetate spots with various surface areas (Experiment No. 1 from Table II). Left: care was taken to form as thin a band as possible at the origin. Right: after the front had reached the upper end of the layer, the chromatograms were left in the tank for 48 h to allow the spots to spread by diffusion. For elution (*cf.* Fig. 4) quadrangles of the same area were used for both compact and diffuse spots.

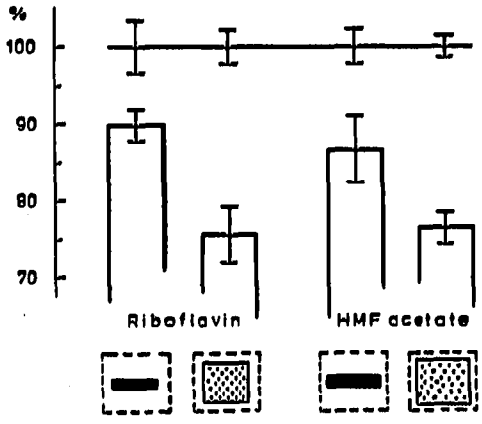


Fig. 4. Difference in recoveries for compact and diffuse spots (Experiment No. 1, Table II; *cf.* Fig. 3). Coefficients of variation are designated by vertical bars.

Influence of the length of run

Longer runs would be expected to cause higher losses owing to the larger amount of sorbent with which the solute has been in contact and because larger spots are produced. Table III shows the results of an experiment devised to

TABLE III

COMPARISON OF RECOVERIES FROM SHORT AND LONG RUNS[a]

Flavin (0.1 *µg*)	*Expt. No.*[b]	*Recovery* (%)		
		Mean ± *S.D.*		*Difference of the means, short run minus long run*
		Short run	*Long run*	
Riboflavin	1	87.3 ± 3.6	87.9 ± 1.3	−0.6
	2	82.8 ± 1.1	78.2 ± 2.2	+4.6
HMF acetate	3	86.9 ± 1.3	84.9 ± 2.0	+2.0

[a] In Experiments 1 and 3, the distance of the flavin spots was three times greater and in Experiment 2 six times greater in the long than in the short run.

[b] Six spots in each group.

illustrate this point. For riboflavin, a statistically highly significant decrease in recovery was caused by increasing the length of the run six times (Experiment No. 2), but even this unusually large difference in the length of the run was responsible for a difference in recovery of only 4.6 %.

Re-solution

In some experiments, the sorbent that was centrifuged off after the first elution was re-suspended in another portion of the eluent, centrifuged again and decanted. These second elutions yielded a recovery of about 5–8 % for riboflavin (after both kinds of TLC) and 2–4 % for HMF acetate on silica gel. The results were widely scattered and when they were added to the recoveries from the first elution of the same spots, the total values did *not* show any improvement in homogeneity (*i.e.*, diminished variation).

CONCLUSIONS

As expected, there was a tendency for lower yields to be obtained for larger spot sizes and longer runs, but these differences were not very marked. It is likely that if the standards were run on the same plate and subjected to similar treatment, minor variations in spot areas and shapes, lengths of runs or R_F values would not cause important errors.

Recoveries were dependent on concentration, decreasing with lower amounts of sample. This factor would be covered by the calibration graph in the actual analytical procedure (fluorescence signal *vs.* amount of sample). It is important that these recoveries did not show higher coefficients of variation than the results of the reference determinations; thus chromatography and elution did not seem to contribute any additional inaccuracy to the variation inherent in the pipetting[23] and fluorimetric procedures.

The results are strictly valid only for the substances and techniques that were studied. However, as the two flavins that were studied are examples of substances of high and intermediate polarity, respectively, and exhibit higher adsorptivity than many other organic compounds, and as the sorbents and the solvent system used were the common ones, the results may serve as illustrations of the degree of accuracy that can be expected in routine TLC fluorimetric methods which are based on partition chromatography and include elution from the sorbent.

REFERENCES

1 F. Snyder and D. Smith, *Separ. Sci.*, 1 (1966) 709.
2 F. Snyder and D. Smith, in R. A. Keller (Editor), *Separation Techniques in Chemistry and Biochemistry*, Marcel Dekker, New York, 1967, pp. 331–344.
3 F. Snyder, *J. Chromatogr.*, 78 (1973) 141.
4 D. E. Jänchen, in E. J. Shellard (Editor), *Quantitative Paper and Thin-Layer Chromatography*, Academic Press, London and New York, 1968, pp. 71–78.
5 E. Sawicki, T. W. Stanley and W. C. Elbert, *J. Chromatogr.*, 20 (1965) 348.
6 H. T. Gordon, *J. Chromatogr.*, 22 (1966) 60.
7 D. Jänchen and G. Pataki, *J. Chromatogr.*, 33 (1968) 391.
8 G. Pataki and K.-T. Wang, *J. Chromatogr.*, 37 (1968) 499.
9 M. Doss, B. Ulshöfer and R. Quast, *J. Chromatogr.*, 41 (1969) 386.
10 R. K. Ibrahim, *J. Chromatogr.*, 42 (1969) 544.

11 H. ZÜRCHER, G. PATAKI, J. BORKO AND R. W. FREI, *J. Chromatogr.*, 43 (1969) 457.
12 V. V. NESTEROV, B. G. BELENKII AND L. G. SENYUTENKOVA, *Biokhimiya*, 34 (1969) 824.
13 L. TÓTH, *J. Chromatogr.*, 50 (1970) 72.
14 B. C. MADSEN AND H. W. LATZ, *J. Chromatogr.*, 50 (1970) 288.
15 A. CIEGLER AND C. P. KURTZMAN, *J. Chromatogr.*, 51 (1970) 511.
16 *Quantitative Chromatogram Analysis*, Schoeffel Instruments Corp., Westwood, N. J., 1969.
17 W. E. COURT, in E. J. SHELLARD (Editor), *Quantitative Paper and Thin-Layer Chromatography*, Academic Press, London and New York, 1968, pp. 29–49.
18 J. CERMAN AND I. M. HAIS, *J. Amer. Chem. Soc.*, 94 (1972) 1741.
19 E. MARKLOVÁ AND I. M. HAIS, *J. Chromatogr.*, 63 (1971) 446.
20 S. UDENFRIEND, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York and London, 1962, pp. 236–241.
21 P. CERLETTI, *Anal. Chim. Acta*, 20 (1959) 243.
22 K. MASŁOWSKI, *J. Chromatogr.*, 18 (1965) 609.
23 J. W. FAIRBAIRN AND S. J. RELPH, *J. Chromatogr.*, 33 (1968) 494.

DISCUSSION

HEATHCOTE: Can you suggest any explanation for the destruction of a flavin on such a mild support medium as cellulose?

HAIS: Hydroxymethylflavin esters are rather labile compounds. This is not surprising in view of their chemistry (substituted aminomethyl esters). They are rapidly converted to lumichrome in alkaline or strongly acidic media and degraded irreversibly by reductants. They undergo rapid irreversible photobleaching, which leads to lumichrome, but they are relatively resistant to light, if air is present. One conjecture would be that the degradation of hydroxymethylflavin acetate was due to the few reducing groups present in cellulose.

PROCHÁZKA: I wonder whether the silicic acid gel could not have protected the labile ester?

HAIS: The ester does not seem to undergo important losses when it is dissolved in the mobile phase of the butanol–acetic acid–water mixture, even in the absence of silica gel.