Journal of Chromatography, 78 (1973) 161–164

CHROM. 6478

PHOTOGRAPHIC QUANTITATION OF FLAT-BED CHROMATOGRAMS USING VARIOUS EXPOSURE TIMES

J. PILNÝ, E. SVOJTKOVÁ*, M. JUŘICOVÁ AND Z. DEYL
Physiological Institute, Czechoslovak Academy of Science, Budějovická 1083, Prague 4-Krč (Czechoslovakia)

SUMMARY

A technique for the quantitation of flat-bed chromatograms or electropherograms involving the minimum exposure time necessary for a spot just not to appear on a photograph has been developed. For calibration purposes the minimum exposure time is plotted against the amount of substance spotted. The amount of a substance present in an unknown spot is determined from the minimum exposure time. The procedure is inexpensive, simple and particularly suitable for occasional quantitations.

INTRODUCTION

The quantitation of flat-bed chromatograms and electropherograms by comparison with a series of standard spots has been developed to an advanced stage (KOLOUŠEK AND COULSON[1]; KOSIKOVSKY[2]; SOUCHON AND GRUNAU[3]; POLSON *et al.*[4]; KIRBY-BERRY[5]). It might be thought that this technique is nowadays slightly outdated as more sophisticated procedures involving the use of computer systems for spot quantitation are becoming more widely used (BUSH[6]; BUSH AND HOFFMAN[7]; SNYDER[8], and references in SNYDER's paper). However, in very complex mixtures (frequently in polyacrylamide gel electrophoretic separations), it may happen that the resolution is insufficient to permit this complex quantitative procedure and also a simple direct comparison of an unknown spot or zone with the standard series may not give adequate results. This is particularly true for close separations or for situations in which a complex separation is obscured by the uneven distribution of individual components in the mixture being analysed. A typical example of such a separation is presented in Fig. 1, in which breakdown products of collagen were separated by polyacrylamide gel electrophoresis. From Fig. 1 it is also obvious that various bands may disappear at various exposure times when photographed and, furthermore, that some bands that originally appear as a single band may be split into two bands under a specified exposure time.

These observations stimulated our efforts to use the minimum exposure, *i.e.*, the time necessary for a particular spot just not to appear, as a measure of the amount of the substance applied to the chromatograms.

MATERIALS AND METHODS

The separation conditions used are specified in Figs. 1 and 2. Generally, the quantitation procedure is applicable to any chromatographic separation that meets

* Department of Radiotoxicology, Uranium Industry Health Institute, Příbram, Czechoslovakia.

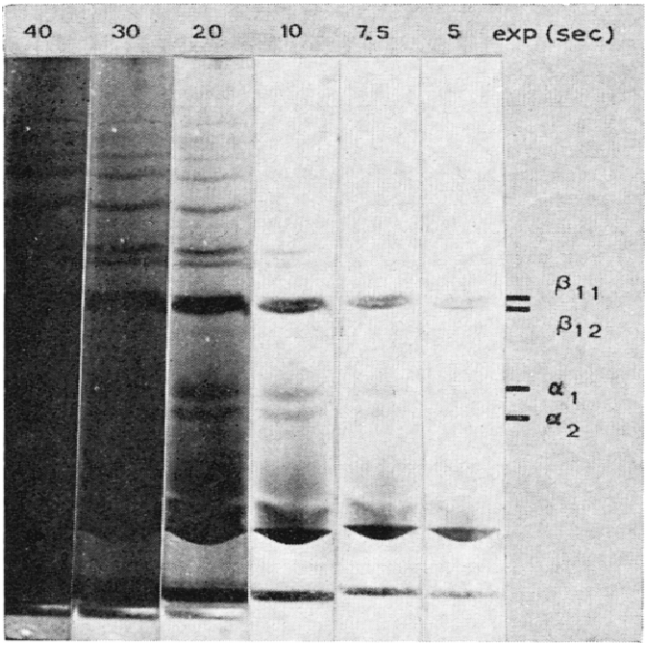

Fig. 1. Typical appearance of separation suitable for quantitation by the developed procedure. Collagen breakdown products were separated in acetate buffer of pH 4.8 on 4% polyacrylamide gel. Gels, 0.6 × 6 cm, were run at 6 mA per gel for 5 h; staining with Amido Black, with electrophoretic de-staining at reversed polarity with 7% acetic acid. The technique used has been described by NAGAI *et al.*[9].

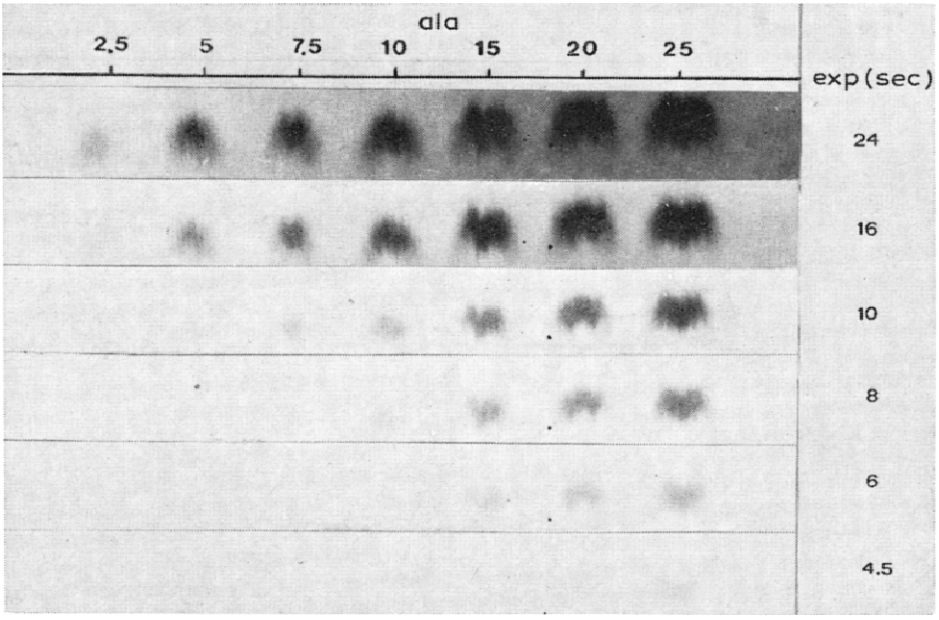

Fig. 2. Stepwise disappearance of seven standard spots of alanine. Separation on Whatman 3 MM filter-paper in *n*-butanol–acetic acid–water (144 : 13 : 43), repeated three times, and staining by the dipping ninhydrin procedure (0.5% ninhydrin in acetone, 24 h at room temperature before exposure). Exposures are given in seconds and amounts applied in micromoles.

the basic rules for quantitation *in situ*. In order to be able to compare the data with those from another chromatographic procedure, mixtures of amino acids analysed by the proposed method were also analysed by routine amino acid analysis with an automated two-column amino acid analyzer (HD 1200 ZSNP, n.p., Žiar n.Hronom, Czechsolovakia).

Photographs of developed chromatograms or electropherograms were taken on NP 15 (ORWO, VEB Fabrik, Wolfen, G.D.R.) film using R9 negative developer and an orange panchromatic filter. In the positive phase, extra hard paper (ORWO, VEB Fabrik, Wolfen, G.D.R.) was used. B109 developer (ORWO, VEB Fabrik, Wolfen, G.D.R.) was used for the positive phase. The photographic system for copying the chromatograms was maintained constant during all of the experiments. The temperature was kept constant at 19–20° during development and the exposure time was pre-set with an automatic timer. Negatives were taken with Exacta equipment from a distance of 40 cm and two sources of 100 W were used for illumination.

A standard series of seven spots (or zones) of increasing concentration was subjected to a series of exposures so that stepwise the seventh, sixth, fifth, etc., spots disappeared (Fig. 2). The minimum exposure times thus estimated were plotted against the amount of substance applied. In this way, a calibration graph was obtained (Fig. 3). The unknown spot was subjected to an identical procedure and from the exposure time necessary for this spot to disappear, the amount of substance present in the spot or zone was determined.

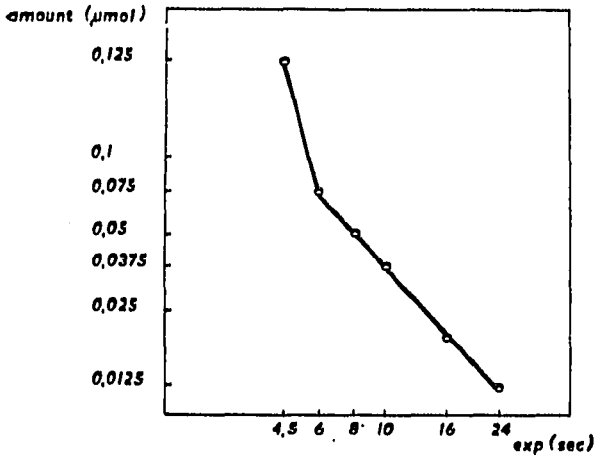

Fig. 3. Example of the calibration graph. The calibration curve is derived from the minimum exposure times observed in Fig. 2. Logarithmic scales on both axes.

RESULTS

We found that this procedure gives quantitative results within a standard error of ±2–4% relative in polyacrylamide gel electrophoresis and a standard error of less than 10% in amino acid analysis. In the test runs with the automated amino acid analyzer, the accuracy limits were ±1.5% (with standard solutions that did not involve hydrolysis of the natural material), which indicated that the described

method maintains a very high level of accuracy. From the practical point of view, this method is very suitable for the quantitation of very complex mixtures of rare materials which do not allow trial runs and which do not offer separations of very good quality. It has also been observed that with transparent beds, such as polyacrylamide gel, it is preferable to estimate the minimum exposure time in the negative phase, while on paper or thin layers of alumina it is preferable to establish the minimum exposure time in the positive phase. It can also be mentioned that the procedure does not require the use of special equipment and is specially useful when occasional quantitation has to be carried out.

REFERENCES

1 J. KOLOUŠEK AND C. B. COULSON, *Chem. Listy*, 46 (1952) 186.
2 F. V. KOSIKOVSKY, *J. Dairy Sci.*, 34 (1951) 235.
3 F. SOUCHON AND G. GRUNAU, *Z. Gesamte Exp. Med.*, 119 (1952) 641.
4 A. POLSON, V. M. MOSLEY AND R. W. G. WYCKOFF, *Science*, 105 (1947) 603.
5 H. KIRBY-BERRY, *Tex. Univ. Publ.*, No. 5109 (1961) 88.
6 I. E. BUSH, *Clin. Chem.*, 14 (1968) 491.
7 I. E. BUSH AND C. P. HOFFMAN, *J. Chromatogr.*, 30 (1967) 164.
8 F. SNYDER, *J. Chromatogr.*, 78 (1973) 144.
9 Y. NAGAI, J. GROSS AND K. A. PIEZ, *Ann. N.Y. Acad. Sci.*, 121 (1964) 494.

DISCUSSION

GOLDMAN: Dr. DEYL, please will you clarify the conditions under which the photography is carried out in your technique. In particular, would you state the spectral distribution and incident direction of the illuminant light, and whether the natural chromophoric properties of the chromatographic zones are used, or whether staining is usually employed. I feel it may be of some importance to maintain a standard form of illumination because, for example, if the plate were illuminated from below rather than above, an increase in contrast of perhaps five times may occur.

DEYL: The system for photographing our chromatograms or electropherograms has been set up once, and remains unchanged. We have used routine techniques of detection such as Coomassie Brilliant Blue for proteins or dipping ninhydrin for amino acids.

HAIS: All-or-nothing methods, such as the determination based on spot area or on detection limit with various exposures on a hard photographic material, that you have presented, are greatly affected by the distribution of mass over the spot surface. The method of spotting of the sample must be very reproducible. In addition, other solutes present in the sample can influence the shape of the spot by displacement or other effects. I should place more confidence in the visual comparison with a standard of known mass, since in this case intensity, area and shape can all be taken into account.

DEYL: I have stressed already that general demands upon quantitation have to be met. In our hands the method presented here was suitable for quantitation of small zones moving closely to very intense zones. Also, very good results were obtained with different types of gel electrophoresis, namely with polyacrylamide gel. In this case the way in which the sample is applied is kept reasonably standard and the possible errors mentioned are minimized.