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HIGH-SENSITIVITY RADIOASSAY IN CHROMATOGRAPHIC EFFLUENTS

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SUMMARY

We have devised convenient systems for fractionating the effluent of high-performance liquid chromatography (HPLC) columns and concentrating the fractions for radioassay. A succession of aliquots of the HPLC effluent is deposited in wells formed in non-wetting fluorocarbon film and evaporated to near-dryness where the aliquots form droplets of uniform size. The droplets are then quantitatively transferred to filter paper impregnated with scintillator, where they form uniform circles 2–3 mm in diameter. Papers containing samples and standards are then exposed to photographic film for a time dependent on the radioactivity of the sample, the film is developed and the autoradiographs are scanned with a thin-layer chromatography scanner used as a densitometer. The density of the spots was proportional to the radioactivity and could be quantified by comparison with the density produced by the standards. Scans of successions of spots from samples collected during elution of amino acids from HPLC columns reproduced the shapes of the peaks recorded by the UV-absorbance detector flow-cell, demonstrating that the resolution of the analysis was preserved. Film blackening sufficient for quantification was obtained with samples containing ^{14}C in the 100–1000 dpm range in as little as 6 h of exposure with further increases in sensitivity in proportion to the increase in time of exposure. Hundreds of fractions from an HPLC effluent and samples from several effluents could be assayed simultaneously, thus offering a large workload capability along with high sensitivity. We postulate that the same technique will produce similarly high sensitivity with little loss of resolution when used in conjunction with methods previously described for stripping radioactive compounds from gas–liquid chromatographic effluents into flowing liquid streams.

INTRODUCTION

Because of the random nature of radioactive decay, the precision of any radioassay is a function of the number of radioactive disintegrations that contribute to the measurement. To be measured with any prescribed level of precision, the sample must therefore remain in the active volume of the detection system for the time necessary for the required number of disintegrations to occur, a time inversely related

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to the radioactivity of the specimen. In radioassays of mixtures resolved by chromatography on columns, either gas–liquid chromatography (GLC) or high performance liquid chromatography (HPLC), preserving the resolution of the chromatography involves assaying a large number of fractions of the effluent. If there is sufficient radioactivity in individual compounds of interest, the radioactivity can be measured by passing the column effluent through a "flow-through" detector, such as the flow cell of a scintillation counter or, in GLC, a flow-through ionization chamber or proportional counter. A record of the counting rate of the detector is similar in appearance to the record of any of the more usual chromatographic detectors. Since the time that portions of the effluent can be permitted to remain in the detector must be limited, if the resolution of the separation is to be preserved, the ability to perform radioassays "on-line", by this approach, is limited to samples with appreciable radioactivity. In radioassays of simple mixtures separated by packed-column GLC, performed in 30–40 min, samples containing at least 30,000 dpm of ^{14}C can be assayed this way, by using flow cells with residence times (volume/flow-rate) of 15–20 sec. Detection of individual compounds with 500 dpm or more is feasible. With slower chromatographic systems, individual portions of the column effluent can be permitted to remain in the active volume of the detector for longer assay times without materially affecting resolution. As a result, somewhat lower levels of radioactivity can be measured. When samples contain appreciably less radioactivity, 1000 dpm or less, assays generally require fractionation of the effluent for subsequent, off-line radioassay in which the time for assaying individual fractions can be extended for additional precision.

The same principles apply to mixtures separated on solid matrices, such as in paper, gel, or thin-layer chromatography (TLC). If radiation is to be detected by a single scanning detector, such as a proportional counter, exposing individual portions of the matrix to the detector for 15–20 sec results in precision that is equivalent to that achieved in assays of column effluents. Radioassay of solid matrices is limited somewhat by the generally lower sensitivity of detection of low-energy beta-particles on solid matrices. This disadvantage is more than counterbalanced by the ability to adjust scanning speed according to the radioactivity present, an approach that is preferable to adjusting the speed of a radioassay to the entirely unrelated speed of the chromatography.

With either kind of chromatography, off-line radioassay requires that a great many samples be assayed, each for an appreciable time period. If a single radiation detector is used, the number of samples and the time required for each severely limits the number of assays that can be assayed in any working period. Absolute sensitivity is also compromised when the levels of radioactivity are close to the background of the radiation detector. Excessively long assay times are often required to distinguish radioactivity in the sample from fluctuation in background counting rate and electronic noise.

Many of the difficulties associated with assaying many samples can be overcome through the use of multiple radiation detectors operating simultaneously. Autoradiography is a relatively inexpensive approach to accomplishing this and, as a result, has come to be preferred to scintillation counting, despite its lower sensitivity, for such applications as radioassay of proteins separated by gel chromatography. In the work described here we devised an approach for using autoradiography in quan-

titative radioassay of effluents from chromatography columns. We explored the use of a novel method for concentrating and evaporating liquids, now used for applying samples to TLC plates, as the basis for a fraction collector for HPLC effluents. We fractionate the effluent, concentrate fractions to small volumes of uniform size, transfer the fractions to a paper strip impregnated with scintillator, and expose the strip to photographic film. After a sufficient time of exposure, the film is developed and scanned with a densitometer.

EXPERIMENTAL

Materials

^{14}C-labeled amino acids and Enlightning Autoradiograph Enhancer were purchased from New England Nuclear (Boston, MA, U.S.A.). Chromatography paper, 1.5 in. × 300 ft. reel, was purchased from Whatman (Clifton, NJ, U.S.A.); the X-ray film, XAR-5, from Kodak (Rochester, NY, U.S.A.); ethylene glycol, Baker grade, from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

Equipment

Samples were collected from the effluent of an HPLC column by directing the outflow of the detector flow-cell to a "Contact Spotter", purchased from Clark Analytical Systems (Sierra Madre, CA, U.S.A.) (Fig. 1).

In the contact spotting technique of applying samples to HPTLC plates, described by Fenimore and Davis[1], many samples contained in relatively large volumes of volatile organic solvents are simultaneously concentrated almost to dryness, following which they are quantitatively transferred to the TLC plate as spots well below 1 mm in diameter. The contact spotter consists of a hollow, rectangular stainless-steel hotplate. Its upper surface contains two rows of 8-mm diameter depressions, 12 mm apart, center to center, and approximately 3 mm deep. At the bottom of each depression is a pin-hole for applying a vacuum. A strip of poly-fluorocarbon film, coated with a fluorocarbon liquid, is placed over the rows of depressions. A vacuum is applied, causing the film to indent into wells at each depression. Samples dissolved in up to 250–300 μl of any of a variety of solvents are deposited into the wells, the plate is covered, and the solvents are evaporated by heating the plate to 50°C and applying a stream of nitrogen. Because of the fluorocarbon coating, the solvent does not wet the film and the samples assume almost spherical shape. As the solvent is evaporated, the samples maintain their spherical shape as they become vanishingly small. When only a small quantity of solvent remains in the bottom of each well, a quantity that is controlled by adding a specified concentration of a less volatile liquid to the solvent, the TLC plate is placed over the wells. The vacuum beneath the film is then replaced by a pulse of pressure greater than atmospheric which everts the wells and transfers the samples to the plate. The contact spotter permits up to 30 samples to be spotted simultaneously. The entire process of evaporation and spotting, which results in quantitative transfer of material and quite uniform spots, requires 5–10 min.

Procedure

To determine the feasibility of using the spotter for preparing fractions for

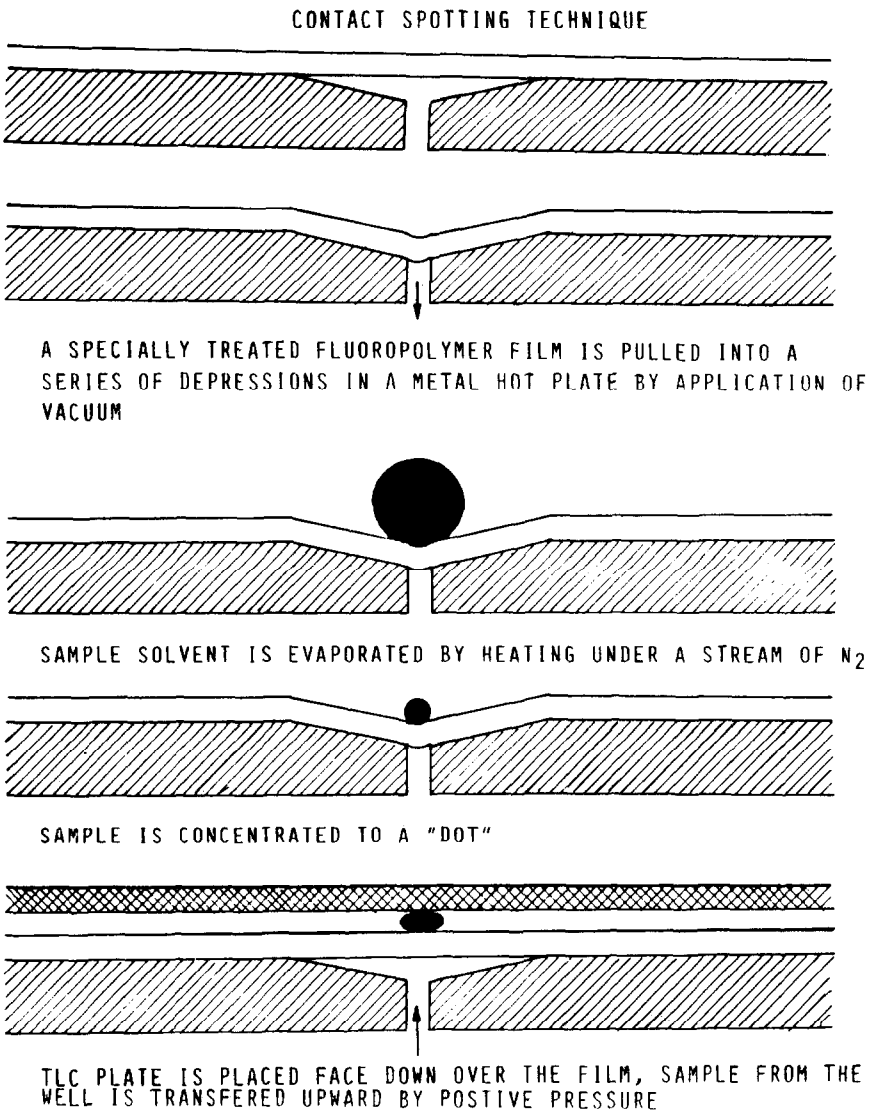

Fig. 1. Diagram of the contact spotting technique.

autoradiography, a 25 cm × 0.5 mm I.D. Teflon® tubing was connected to the outlet of the HPLC detector flow cell with its end suspended over the contact spotter. Approximately 350 μl of column effluent was deposited in each well. Two μl of 1% ethylene glycol in mobile phase were added to each well and the fractions were evaporated to "dryness" at 50°C. The spots were then transferred to a 5 cm wide strip of chromatography paper previously dipped into Enlightning Autoradiography Enhancer scintillation solution. A radioautogram of the paper was then prepared by clamping the paper against a sheet of X-ray film in a Wolf X-ray cassette for exposure times of 6–15 h. The film was then developed and the density of the spots was scanned

with the chromatogram scanner, Model CS-910 (Shimadzu, Columbia, MD, U.S.A.) operated in the transmission mode, with a slit width set at 0.5 mm, a slit length at 3.5 mm, and a scan speed of 40 mm/min. The scanner signal was processed on an HP-3390A Integrator (Hewlett Packard, Avondale, PA, U.S.A.). The liquid chromatograph consisted of two Altex 110A pumps and a Model 420 gradient microprocessor (Altex Scientific, Berkeley, CA, U.S.A.) set up to analyze amino acids by a procedure described previously[2] involving mixed chelate complexation of the amino acids with copper sulfate. The elution of the amino acids was monitored by recording the absorbance of the copper complexes at 234 nm with a Spectroflow 757 UV detector (Kratos Analytical Instruments, Ramsey, NY, U.S.A.), connected to a Model 4416 data system (Nelson Analytical, Cupertion, CA, U.S.A.) and a Model 56 strip chart recorder (Perkin-Elmer, Norwalk, CT, U.S.A.)

Standardization procedure

Standards of [^{14}C]proline containing 4000, 2000, 1600, 1000, 800, 400, 200,100, 80, 40 and 20 ^{14}C dpm/μl were prepared by diluting a stock solution (New England Nuclear, Boston, MA, U.S.A.) with 2.0 $\times$ 10^4 dpm/μl in mobile phase buffer. A 10-μl aliquot of each standard and 150 μl of the mobile-phase buffer containing copper sulfate and ethylene glycol was deposited on the contact spotter and evaporated to a final volume of approximately 50 nl. The samples were then transferred to the paper strip impregnated with Enlightning® and a radioautogram was prepared and scanned as above.

RESULTS

Autoradiograms of graded quantities of [^{14}C]proline standard showed blackening adequate for scanning after a 6-h exposure (Fig. 2). Because of the presence of copper sulfate in the mobile phase, the size of the individual spots on the paper could easily be seen and determined to be uniform. The diameter of the spots on the film were not detectably larger than those of the spots on the paper, reflecting the close

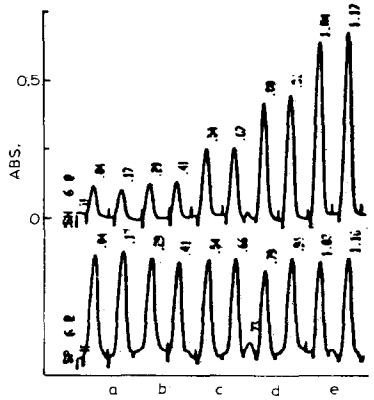

Fig. 2. Upper curve: scan of autoradiographs of graded quantities of [^{14}C]proline, 6 h exposure, a = 800, b = 1000, c = 2000, d = 4000, e = 8000 dpm, each in duplicate. Lower curve: Reflectance scans of spots, primarily attributable to the copper sulfate in the mobile phase, on chromatography paper.

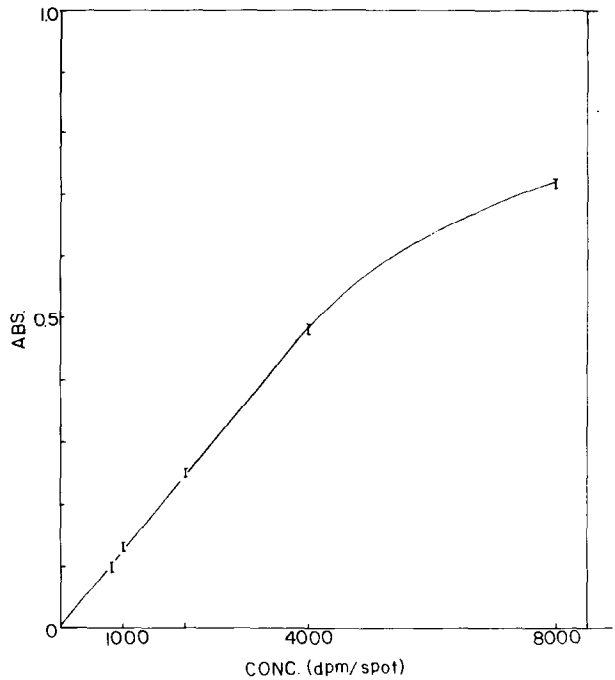

Fig. 3. Standard curve of scans of [^{14}C]proline radiographs after 6 h exposure.

contact between the paper and the film. A standard curve for 6-h exposure, obtained by plotting the absorbance *vs.* radioactivity in dpm showed proportionality up to 4000 dpm, which produced a spot with absorbance of 0.55 (Fig. 3).

Lower radioactivity was detected by extending exposure time to 15 h (Fig. 4). The absorbance was again directly proportional to dpm within the linear range of the X-ray film (Fig. 5). Samples containing 20–80 dpm were readily detected after 15-h exposure (Fig. 6). The coefficient of variation of the scans of the autoradiographs obtained by depositing 6 samples, each containing 80 dpm of ^{14}C was 7.5% (Fig. 7).

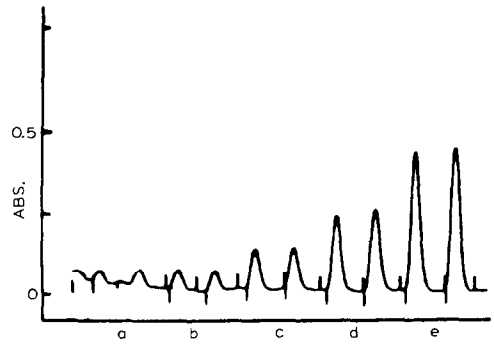

Fig. 4. Scans of autoradiographs of graded quantities of [^{14}C]proline after 15 h exposure: a = 80, b = 200, c = 400, d = 800, e = 1600 dpm, each in duplicate.

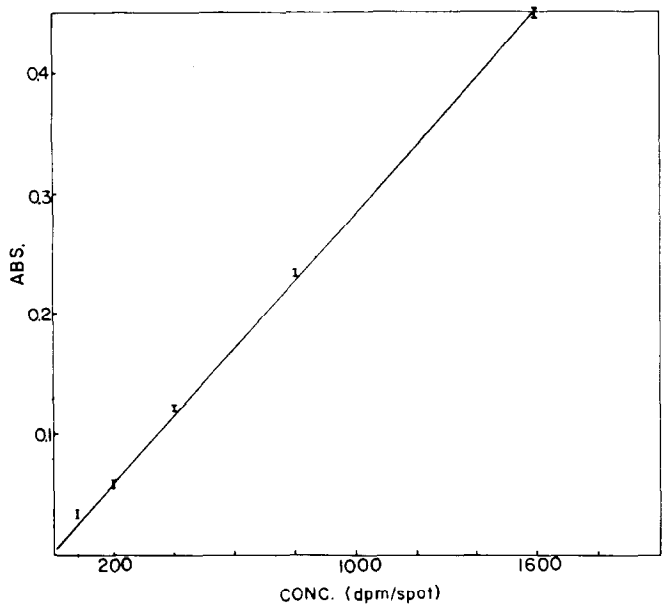

Fig. 5. Standard curve of scans of [^{14}C]proline radioautographs after 15 h exposure.

To assess the feasibility of using the contact spotter for collecting fractions, a simple mixture of ^{14}C-labeled amino acids was chromatographed on a reversed-phase column, and equal portions of the effluent were collected on the spotter, ethylene glycol was added to each, the mobile phase was evaporated and transferred to the impregnated paper strip. Because of the limited number of wells on the contact spotter, three series of fractions were collected, corresponding to the periods of elution of the solvent front and the three amino acids, and the flow of mobile phase was stopped between each series of collections. The scans of the radioautographs (Fig. 8) reproduced the shape of the peaks recorded by the UV-absorbance detector. Appreciable radioactivity was also eluted with the solvent front (Fig. 9).

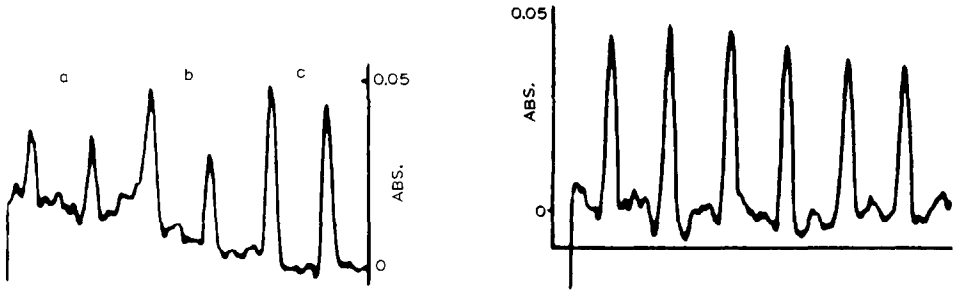

Fig. 6. Scans of autoradiographs of low radioactivity standards after 15 h exposure: a = 20, b = 40, c = 80 dpm, each in duplicate.

Fig. 7. Reproducibility of scans of autoradiographs of 80 dpm samples.

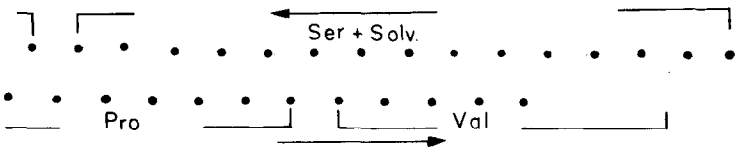

Fig. 8. Radioautographs of fractions collected from an HPLC analysis of a solution containing three [^{14}C]amino acids.

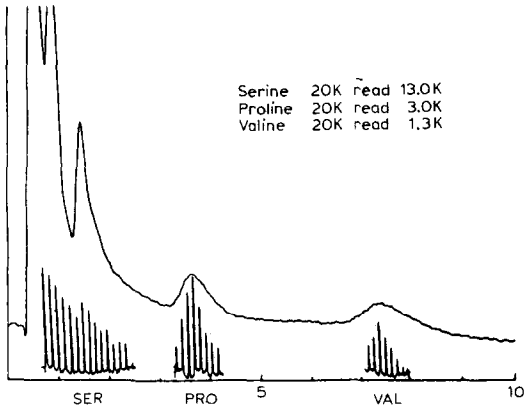

Fig. 9. Scans of the radioautographs in Fig. 7 superimposed on the record of the analysis produced by the UV-absorbance detector at the same times the fractions were collected. During the elution of the first amino acid, serine, 160 μl fractions were collected. During the elution of the second two amino acids, proline and valine, 350 μl fractions were collected.

DISCUSSION

Since the sensitivity of any radioassay depends almost directly on the time allowed for radioactive disintegrations to occur within the sensitive volume of the detector cell, measurement of radioactivity in the effluent of a chromatography column on-line has markedly limited sensitivity. The high speed of modern column chromatographic separations, and the requirement to preserve as much of the resolution of the analysis as possible, limit the time that any portion of the effluent can be permitted to remain in the detector cell. This time, which can be estimated from the ratio of the volume of the detector to the flow-rate, must be limited to 10–15 sec in GLC and can be increased only slightly for HPLC. On-line measurement of radioactivity, which is convenient because the result is available at the conclusion of the analysis, is therefore limited to samples that contain approximately, 30,000 dpm or more, and no less than 500 dpm in the component with the lowest radioactivity. For samples containing appreciably lower radioactivity, fractionation of the effluent for off-line measurement of radioactivity is required. With off-line measurement the time for assaying individual ffractions can be extended and additional precision can be obtained.

The same principles apply to mixtures separated on the solid matrices of paper, gel, and thin-layer chromatography. If radiation is to be detected by a single scanning

detector, such as a proportional counter, exposing individual portions of the matrix to the detector for 15–20 sec results in precision that is equivalent to that achieved with detectors with similar residence times in assays of column effluents. The sensitivity of detection of low-energy beta-particles on solid matrices is limited somewhat by absorption of the beta-particles, but this disadvantage can be more than counterbalanced by the ability to adjust scanning speed according to the radioactivity present. This approach allows a useful trade-off of time for sensitivity and is to be preferred to attempting to adjust the speed of the radioassay to the speed of the chromatography, which is entirely unrelated to the sensitivity of radioassay required.

Just as is the case with detectors that measure an optical or electronic property of a column effluent or on a solid paper or TLC matrix, the reliability of a radioassay is greatest when the distribution of radioactivity is monitored rather than the concentration of the radioactivity in preselected portions of the effluent. For example, in the record shown in Fig. 9, assay of radioactivity in the effluent containing the first amino acid to be eluted from the column included appreciable radioactivity from species that were eluted with the solvent front. Many such examples are encountered that suggest the advisability of attempting to preserve as much of the resolution of the chromatography in the radioassay as possible. One would not generally consider quantifying a component eluted from the HPLC column by collecting a portion of the effluent and measuring its UV absorbance without recording the UV absorbance of similar-sized portions of the effluent collected both before and after the elution of the compound of interest. Radioassay should be performed with similar theoretical considerations. Since radioactivity is not necessarily related to any other physical property of the column effluent, collection of samples based on the response of any other type of GLC or HPLC detector is, similarly, not justified.

Resolution is best preserved and off-line radioassay is most reliable when the radioactivity of a number of equal portions of the effluent is assayed. Off-line radioassay thus requires that a great many samples be assayed, each for an appreciable time period. If a single radiation detector is used, the large number of samples and the time required to assay each severely limit the number of assays that can be performed in any time period. Since background counting rates of most sensitive detectors are generally appreciable, longer counting times are required when the levels of radioactivity are close to the background of the radiation detector in order to distinguish radioactivity in the sample from fluctuation in the background noise.

Radioassay of many samples is aided by the use of multiple detectors, all acting simultaneously. One approach to this has been the use of spark-chambers for assessing the distribution of radioactivity on paper chromatograms and TLC plates. An appreciably simpler approach is the use of autoradiography on photographic film.

The sensitivity of "contact-print" autoradiography of fractions collected from column effluents depends on detecting differences between the film density produced by the radioactivity in individual samples and fluctuations in the density of the film itself. Sensitivity thus depends on the concentration of radioactivity exposed to the film: *i.e.* dpm per unit area. Quantification by densitometry is easier if individual fractions are collected into fairly uniform areas. Both these requirements are perhaps better met with the technology used with the contact spotter than by most other methods for collecting samples. With this technique, relatively large volumes of col-

umn effluent, or other solvents containing the compounds of interest, can be concentrated to small spots of uniform size with a minimum of difficulty. The size of the spots can be controlled by addition of less volatile compounds to the solvent prior to evaporation. We have used decanol for this purpose when concentrating lipid solvents for TLC and ethylene glycol for concentrating the mostly aqueous effluent of reversed-phase HPLC. Adding ethylene glycol permits evaporation to be limited so that the concentration of salt is less than that at which precipitation occurs and thus aids in the transfer of the radioactive compounds to the paper.

We consider the work described here to be a feasilibity study and demonstration of principle rather than a definitive working model for a radioassay system. We have not attempted to maximize the sensitivity of detection of radioactivity on the film by use of high-speed film, by concentrating spots to minimum size, or by most effective use of scintillators to enhance the light output and the sensitivity. Comparison of results obtained by depositing identical quantities of radioactive amino acids on paper untreated with "enhancer" resulted in appreciable detection of the radioactivity. This indicates that much of the film blackening we have observed is attributable to direct interaction of the beta-particles with the film rather than with the scintillator. Previous experience has shown that losses from absorption of the beta-particles on the paper can be reduced, and appreciably increased sensitivity can be obtained, by supplying sufficient scintillator to absorb most of the energy of the beta-particles.

We also have not fully explored the possibility of interference from chemiluminescence or light emission by other mechanisms which are sometimes observed for considerable periods when compounds are concentrated from organic solvents and presumably could contribute to film darkening. The presence of blank areas on the paper between samples provides a baseline for the film from which to measure the blackening caused by the sample and thus aids in quantification by densitometry. We were able to achieve good proportionality between density above baseline and the quantity of radioactivity applied, which attests to the efficacy of the densitometer for quantifying autoradiography as well as to the quantitative and uniform transfer of materials from the fluorocarbon film of the contact spotter to the paper.

If interference from other sources of light emission by the sample proves to be of little quantitative significance, sensitivity could presumably be increased in direct proportion to the time allowed for exposure of the film to the sample. Overnight exposure, as described here, permitted detection of samples that were hardly above the background level of our liquid scintillation counter, provided that the samples were concentrated to spots of only several millimeters in diameter.

The sensitivity obtained so far, which we believe is far from that achievable by an optimized system, is nevertheless appreciably greater than that achieved in the past by a variety of sensitive liquid scintillation techniques for performing similar assays. We are optimistic that it can be the basis of techniques for using chromatography in conjunction with low-energy beta-emitters in biological experiments in which the specific radioactivities have been too low to be measurable with previously available technology. Although, the work described here was limited to radioassay of HPLC effluents, we have every reason to believe the technique to be equally applicable to GLC. We have performed many studies in which compounds eluted from GLC columns with gas flows of 40–50 ml/min were stripped continuously into 1–2

ml of organic solvent for subsequent measurement of fluorescence or UV absorbance with little loss of resolution[3,4]. We have also devised methods for burning the effluents of GLC columns to carbon dioxide and water and for collecting the combustion products into flowing alkaline streams for radioassay[5]. Fractionating and concentrating these effluents, which are relatively uniform in composition, for autoradiography should be even simpler than collecting and concentrating fractions of HPLC effluents that have different composition, salt content, and volatility.

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