CHROM. 6255

A MINIATURE FLOW FLUOROMETER FOR LIQUID CHROMATOGRAPHY

LOUIS H. THACKER
Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, Tenn. 37830 (U.S.A.)*
(Received June 19th, 1972)

SUMMARY

A miniature flow fluorometer has been developed for use as a detector in liquid chromatograph systems. Excitation wavelengths can be chosen among the low-pressure mercury lines by a choice of filters; emission wavelengths between 310 and 650 nm can be observed, and the accepted emission spectrum can be limited by blocking filters. The instrument is presently in use in the Body Fluids Analyses Program of the Oak Ridge National Laboratory in a new system developed to monitor classes of compounds previously difficult to detect in chromatograph eluates. In this service the new instrument will detect well-separated organic acids present to the extent of 100–500 ng in the injected sample.

INTRODUCTION

High-resolution liquid chromatography is extensively used as the separation step in the analysis of chemically complex solutions, *e.g.*, physiologic fluids such as urine and blood serum[1,2]. Miniature photometers are available for detection of compounds which are UV absorbers and for those which absorb visible light, either directly or after reaction with color-developing reagents[3,4].

There are also classes of compounds which exhibit either intrinsic fluorescence or fluorescence resulting from a developing reaction; when such fluorescence is available it permits analytical measurements more sensitive than those of photometry by a factor usually exceeding 1000 (refs. 5 and 6). Some examples include liquid chromatographic studies involving a ninhydrin reaction with primary amines and aldehydes[7,8] and a cerate oxidative reaction with organic acids and other compounds[9].

The miniature flow fluorometer reported here was developed in response to the needs of researchers in the Body Fluids Analyses Program of the Oak Ridge National Laboratory who were developing the new cerate oxidation technique for monitoring liquid chromatograph elutes[9]. The early experiments in developing the new monitoring system were conducted using a commercial flow fluorometer; however, the need for increased sensitivity, a miniature detector that could be mounted in close proximity to the column, and a low-cost, committed detector that could be a permanent part of the analytical system led to the development of this instrument. In the present application, the instrument detects fluorescent

* Operated for the U.S. Atomic Energy Commission by the Union Carbide Corporation.

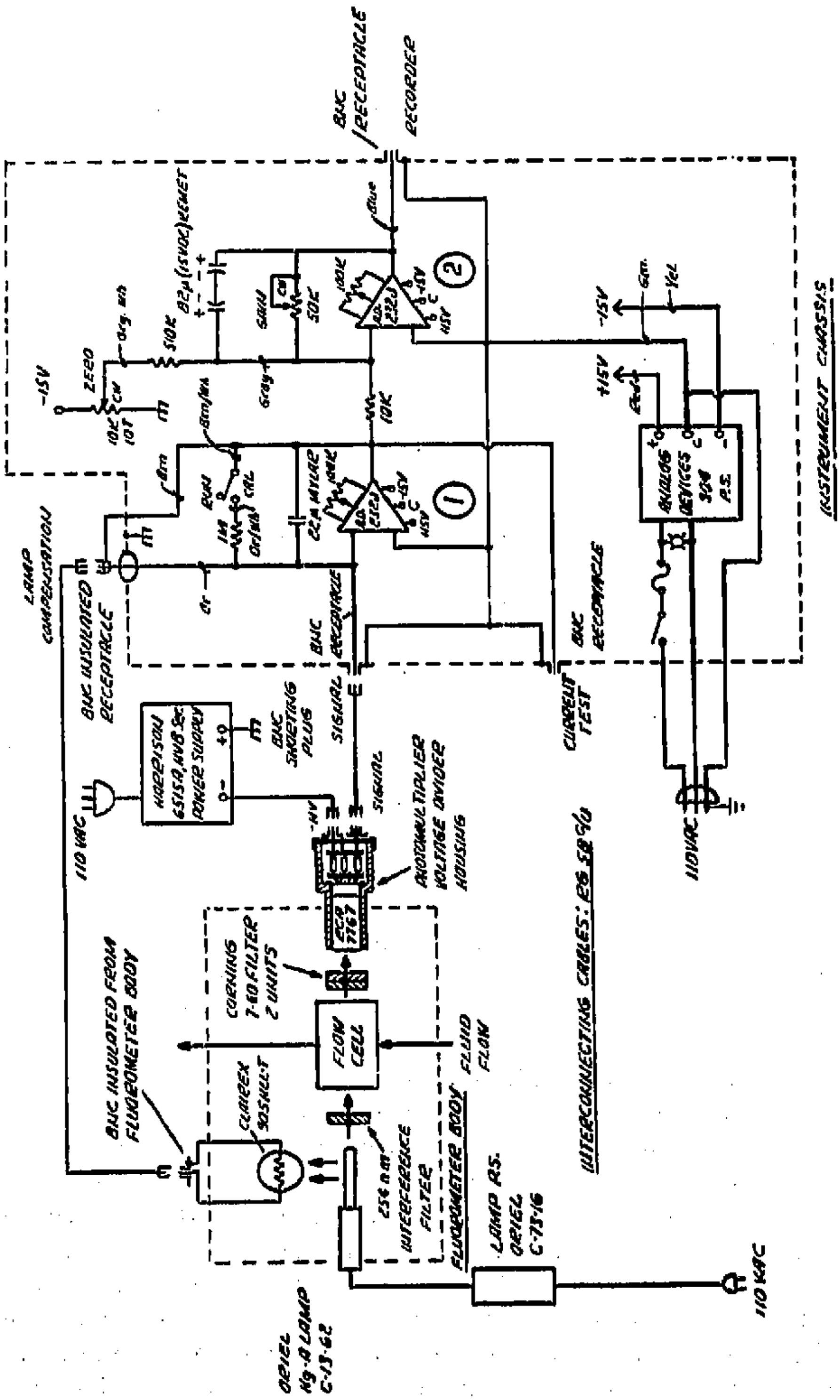

Fig. 1. System diagram.

cerium (III) generated by sample materials that react with reagent cerium(IV) added to the column eluate.

EQUIPMENT DESCRIPTION

The miniature flow fluorometer system (Fig. 2) consists of the fluorometer body and a chassis for electronic components and circuits. The instrument system is shown schematically in Fig. 1.

The fluorometer body is a machined aluminum block (Figs. 1 and 3) which contains a low-pressure mercury lamp, appropriate excitation filter, quartz tube flow cell, appropriate blocking filter, photomultiplier housing, and a photoconductor which compensates for changes in lamp intensity. The mounting accommodations for all of these components are machined into the aluminum block along with the required optical apertures, so there is no problem in establishing or maintaining proper alignment of components. The fluorescence from the sample is observed at a right angle to the excitation radiation.

The low-pressure mercury lamp (Oriel Optics Corp., Model C-13-62) dissipates approximately 4.6 W with approximately 4% conversion efficiency for emission of the mercury spectral lines at 253.7, 312.5, 365, 404.7, 435.8, 546.1, and 577 nm. Of the total irradiance, approximately 90% is in the 253.7-nm line, with the remainder divided approximately equally among the next five longer wavelengths. An Oriel C-73-16 power supply drives the mercury lamp.

The excitation filter can be chosen to select any one, or a group, of the available spectral lines for exciting the sample. Because the excitation maximum for cerium(III) ions is at 260 nm, the instrument is presently being operated with a 254-nm interference filter. The choice of a particular interference filter for this

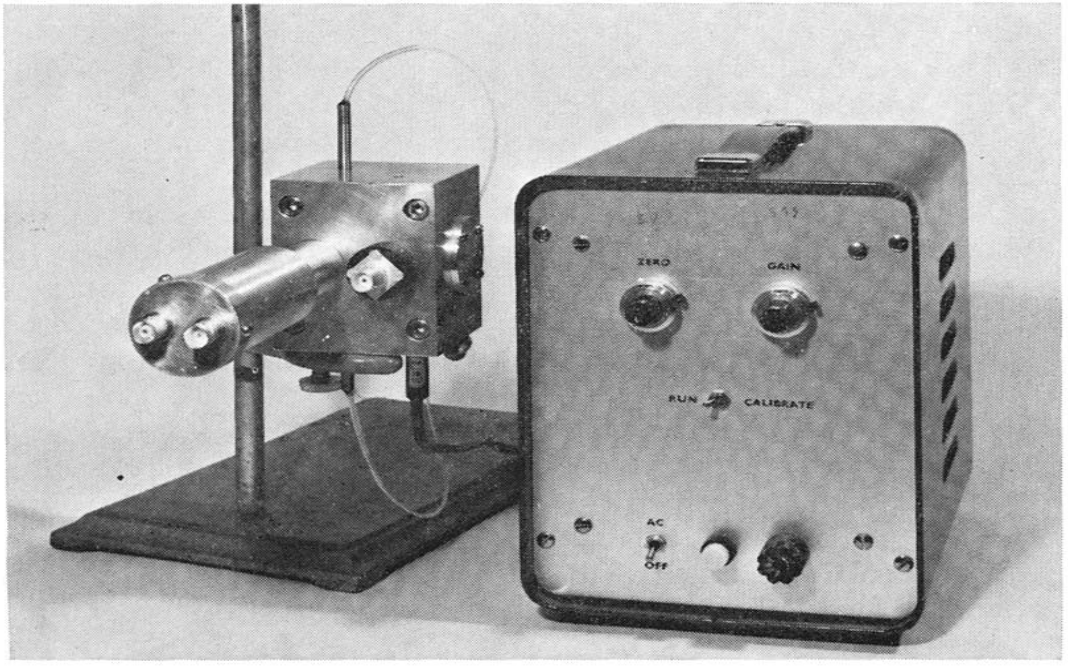

Fig. 2. The miniature flow fluorometer.

service should be based on high transmittance in the pass band and high blocking (10^{-4} to 10^{-5}) at longer wavelengths, rather than on a narrow pass band. Appropriate filters are available from a number of manufacturers.

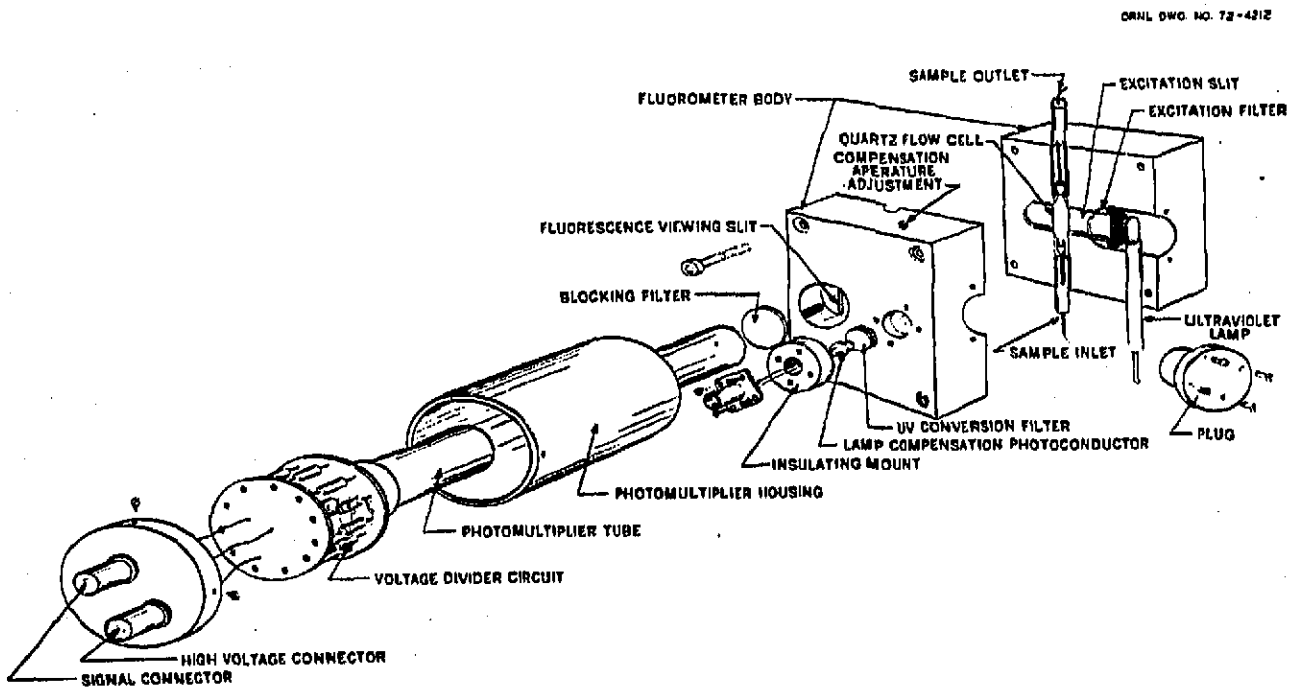

Fig. 3. Fluorometer body.

The flow-cell is a length of 4-mm-O.D. by 0.5-mm-wall quartz tubing 2.5 cm long, with ends pulled down to 1.6 mm O.D.; the total length is 5.7 cm. Each end of the flow cell is connected to a 1.6-mm-O.D. PTFE tubing. The quartz-to-PTFE connection is made by etching the end of the PTFE tube with commercial etchant, abutting the PTFE tube to the quartz tube, covering the joint with a short length of 1.6-mm-I.D. PTFE, and sealing the joint by shrinking a sleeve of shrinkable PTFE over it. These flow-cell assemblies are tested for leaks at elevated pressure before they are installed in the instrument.

The blocking filter which defines the band of wavelengths passed to the photomultiplier can, like the excitation filter, be selected from a wide range of choices. Since the fluorescence emission spectrum of cerium(III) corresponds almost precisely with the pass band of the Corning 7–60 filter, that material was used in the present experiments. Optimum results were obtained with two layers of the 7–60 filter.

The photomultiplier is an RCA 7767 which has an S-11 photoresponse characteristic. This response spectrum imposes the ultimate 310- to 650-nm limit on the detectable fluorescence emission spectrum. The voltage divider for the photomultiplier is contained in the photomultiplier housing, so that only one coaxial high-voltage cable and a signal cable are required.

There is a Clairex CL 905HLL-T photoconductor which senses the 254-nm emission of the mercury lamp through an Instrument Specialties Co. (ISCO), UV conversion filter (ISCO Part No. UA-120). This detector adjusts the gain of

the electronic circuit to compensate for variations in lamp intensity. An aperture-adjusting screw determines the amount of light falling on the photoconductor; the aperture screw is adjusted initially so the photoconductor resistance is 1 MΩ. Subsequent checks and adjustments can be made during normal operation through use of the RUN-CALIBRATE switch on the electronic chassis.

The high voltage for the photomultiplier is supplied by a Harrison Laboratory (Hewlett-Packard HVB Series, Model 6515A) high-voltage power supply.

The principal components in the electronics chassis are a ±15-V d.c. power supply (Analog Devices 904) and two high-quality chopper-stabilized operational amplifiers (Analog Devices 232-J).

Amplifier 1 is operated as a current-to-voltage converter; the signal current from the photomultiplier is taken directly to the summing junction of the amplifier, and the photoconductor in the fluorometer head is connected by a cable as the feedback resistor. Thus, when the intensity of the mercury lamp decreases, the signal current also decreases proportionately; but since the resistance of the photoconductor increases proportionately, the output of amplifier 1 remains essentially constant. The resistance of the compensating photoconductor can be checked while the system is operating by switching the toggle switch on the front panel from the RUN to the CALIBRATE position. In the CALIBRATE position the photoconductor is paralleled by a 1-MΩ resistor; since the desired resistance of the photoconductor is 1 MΩ, the voltage observed at the CURRENT TEST receptacle with the switch in the CALIBRATE position should be exactly half the value with the switch in the RUN position. If this is not the case, the photoconductor resistance is adjusted by the aperture screw at the top of the fluorometer body.

Amplifier 2 provides zero and gain adjustments for interfacing with a variety of standard recorders. Because workers in our programs want to record peaks in the same format (and often on the same chart) as transmittance and absorbance peaks from other instruments, we use zero adjustment to place the base line at positive full scale on the recorder, and fluorescence peaks are recorded as down scale (negative) deflections of the recorder pen. In situations not demanding ultimate sensitivity, amplifier 2 could very probably be an inexpensive integrated circuit amplifier.

Unless line voltages are clean and stable it has been necessary to provide power through a SOLA transformer (Catalog No. 23-22-112-2; 120 VA) for operation at high sensitivities. Since the capacity of the SOLA transformer is adequate, the power to the high-voltage power supply and the mercury lamp power supply is also taken from that source.

EXPERIMENTAL RESULTS[9]

In operational tests in a newly developed liquid chromatograph monitoring system the miniature flow fluorometer is routinely detecting low microgram quantities of organic acids (*e.g.*, lactic, pyruvic, malonic, and α-ketoglutaric) not detectable with UV absorbance photometers. Many compounds other than organic acids are detected, as illustrated in the chromatogram for a urine sample shown in Fig. 4. For that sample, the fluorometer used with the new cerate oxidative monitoring system is more sensitive than the reference UV monitor for a large

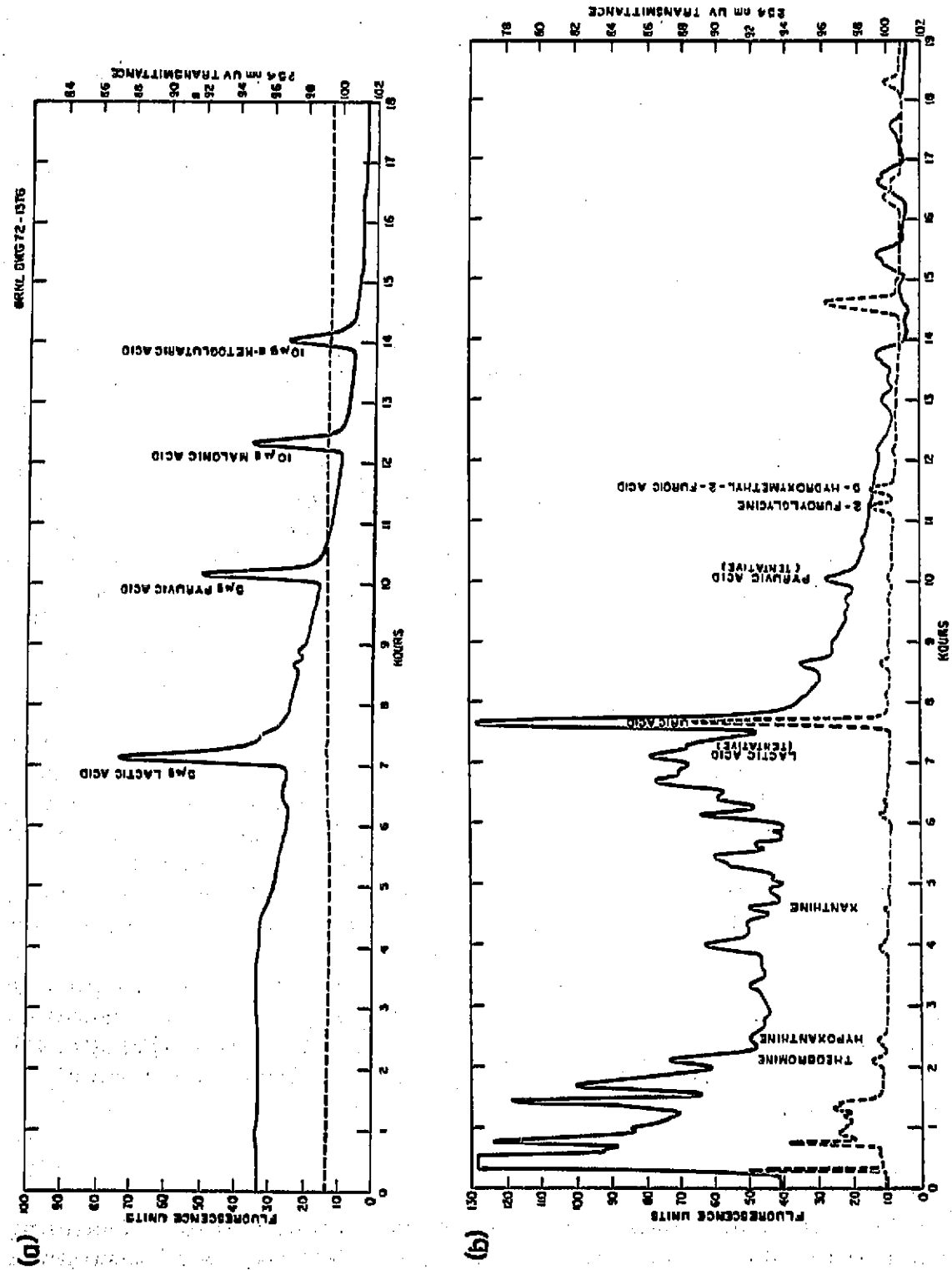

Fig. 4. Test results. (a) Comparison of the detection of organic acids by fluorescence and UV monitors. Elution from a 50-cm long × 4.6-mm-I.D. column of UV analyzer. (b) Comparison of the detection of compounds in urine by fluorescence and UV monitors. Elution from the same column as mentioned under (a). ——, Fluorescence; - - - -, UV.

number of compounds; among those identified are xanthine, theobromine, and uric acid.

The limit of detection for well-separated organic acids appears to be of the order of 100 to 500 ng per injected sample.

ACKNOWLEDGEMENTS

The flow fluorometer was developed in response to the needs of S. KATZ and W. W. PITT, JR., who developed the new cerate oxidative monitoring system. The experimental results quoted here were obtained by them in research sponsored by the National Institute of General Medical Sciences and conducted at the Oak Ridge National Laboratory (operated for the U.S. Atomic Energy Commission by the Union Carbide Corporation).

REFERENCES

1 C. D. SCOTT, J. E. ATTRILL AND N. G. ANDERSON, *Soc. Exp. Biol. Med. Proc.*, 125 (1967) 181.
2 C. D. SCOTT, *Clin. Chem.*, 14 (1968) 521.
3 L. H. THACKER, C. D. SCOTT AND W. W. PITT, JR., *J. Chromatogr.*, 51 (1970) 175.
4 L. H. THACKER, W. W. PITT, JR., S. KATZ AND C. D. SCOTT, *Clin. Chem.*, 16 (1970) 626.
5 G. K. TURNER, *Med. Electr. News*, 3, No. 2 (1963) 16.
6 M. GURKIN AND E. A. KALLET, *Amer. Lab.*, 3, No. 10 (1971) 23.
7 K. SAMEJIMA, W. DAIRMAN, J. STONE AND S. UDENFRIEND, *Anal. Biochem.*, 42 (1971) 273.
8 P. M. FRAZIER AND G. K. SUMMER, *Anal. Biochem.*, 44 (1971) 66.
9 S. KATZ AND W. W. PITT, JR., *Anal. Lett.*, 5, No. 3 (1972) 177.