Journal of Chromatography, 324 (1985) 333–342
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 560

REACTION DETECTOR FOR LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL GENERATION AND DETECTION OF EXCESS BROMINE

KARIN ISAKSSON*, JÖRGEN LINDQUIST* and KENT LUNDSTRÖM**
Department of Analytical Chemistry, University of Uppsala, Box 531, S-751 21 Uppsala (Sweden)
(Received January 15th, 1985)

SUMMARY

A reaction detector based on bromine consumption for liquid chromatography is presented. Bromine is electrochemically generated on-line in a separate flow, which combines in a post-column mode with the column effluent. After the passage of a single-bead-string reactor, with 10–20 sec in residence time, the bromine concentration is monitored by reductive amperometry. Bromine-reactive substances will cause a decrease in amperometric response in proportion to concentration, and an indirect quantification is thus achieved. Detection capabilities are illustrated for methyl-, ethyl- and propylamine, reduced and oxidized glutathione and lysozyme and cytochrome *c*. Depending on reaction conditions, linear dynamic ranges are between one and three orders of magnitude. Representative detection limits are 0.5–10 picomoles with use of conventional liquid-chromatographic equipment.

INTRODUCTION

Important extensions in the scope of presently available detectors for liquid chromatography (LC) can be realized when reaction detection is utilized. Such approaches, normally involving some type of on-line post-column conversion of the target analyte to improve its detectability, have attracted increasing interest during recent years[1–3]. The issue of optimal reactor design has also been addressed[4–7]. Besides the normal concept of direct detection of the converted analyte, indirect reaction detection can be accomplished by monitoring the concentration of a reagent. As illustrated in recent applications with optical detectors[8–11], such indirect approaches offer further means to achieve detectability.

A reaction detector system based on bromine consumption has been presented by King and Kissinger[12]. The bromine is electrochemically generated and its concentration is amperometrically monitored subsequent to the passage of a reactor. Hence, the detector baseline corresponds to the full amperometric response under conditions of no bromine consumption. The generation of bromine can either be

* Present address: Astra Läkemedel AB, S-151 85 Södertälje, Sweden.
** Present address: Kabi Vitrum AB, S-112 87 Stockholm, Sweden.

0021-9673/85/$03.30

effected on-line, which requires the presence of bromide in the mobile phase, or in a separate flow, which is combined with the mobile phase in a post-column mode. As illustrations of detector performance, all involving on-line generation, determinations of unsaturated fatty acids, prostaglandins and phenols were shown.

In the present article, we report on the approach of bromine generation in a separate flow. This is clearly the more versatile of the two approaches in that the choice of adequate conditions for the chromatographic separation as well as for the bromine reaction is facilitated. Despite the inevitable increase in system complexity, the detection limits achievable compare well with those observed with bromine generated on-line.

The detector system is described in detail with our experiences from optimizing the signal-to-noise ratio. The signal is maximized by considering the demands of the bromine consumption reaction primarily on pH and time, while the noise is minimized in terms of the best possible flow stability and optimal generation of bromine. To illustrate the analytical performance, the determinations of primary aliphatic amines, glutathione in reduced and oxidized form, lysozyme and cytochrome *c* are reported. These are examples of compounds that often pose problems with the common ultra-violet, fluorescence and electrochemical detectors, but are suitable for the bromine-reaction detector.

EXPERIMENTAL

Chromatographic equipment

This comprised a screw-driven syringe-type pump (Model 314, ISCO), an injection valve equipped with a 20-μl sampling loop (Model 7120; Rheodyne) and columns as specified for each application *viz.*, MPLC cartridge column system with a 5-μm Spherisorb RP-18 column of size 4.6 × 100 mm (Brownlee Labs.) and strong cation-exchange column Mono S HR 5/50 (Pharmacia, Uppsala, Sweden). An adjustable bellows-valve pulse dampener (Model 709, LDC) was inserted in a T-configuration between the pump and the injector. A by-pass loop, detailed elsewhere[13], with a split ratio of 10:1 was connected in parallel with the injector. The injector and the column were connected by 5 cm of 0.18 mm (0.007 in.) I.D. stainless-steel tubing. The column and the T-connector for post-column reagent mixing were connected by *ca.* 5 cm of PTFE tubing (0.15 mm I.D.). T-connectors for the by-pass loop and the post-column mixing had an internal bore of 0.25 mm (Valco).

Reaction detection system

The flow-through bromine generator cell was fed with the reagent solution from a poly(vinyl chloride) reservoir pressurized from the outside with nitrogen ("pulse-free pump"); Pye Unicam, Cambridge, U.K.). The flow-rate was measured by means of a variable-area flow-meter (Gilmont). The two-electrode generator cell (Fig. 1A) was of the thin-layer type and constructed in KEL-F, with the working electrode and the auxiliary electrode compartment positioned directly opposite each other, their distance being defined by a 100-μm thick PTFE spacer. The working electrode was a 0.8-mm^2 platinum disc, obtained by force-fitting a platinum wire (1 mm in diameter) through an undersized hole and making it flush with the KEL-F block by sanding and polishing the entire surface of the cell half. The final polish

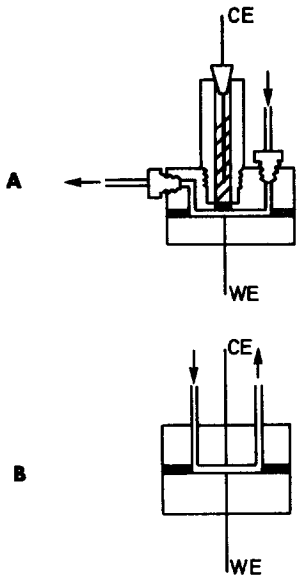


Fig. 1. (A) Generator cell with isolated counter electrode. (B) Two-electrode detector cell.

was carried out with 0.25-μm diamond paste. A porous glass frit (Vycor) was mounted in a similar manner in the other cell half, providing a separate cell compartment for the platinum auxiliary electrode and its electrolyte (3 *M* sodium nitrate). The circuitry of the constant-current source for bromine generation was of conventional design based on integrated-circuit operational amplifiers providing a current range from 0.1 μA to 1 mA. The constant-current circuitry was powered from a voltage supply of ± 15 V.

The reagent stream and the column effluent were combined in a 0.25-mm bore T-junction (Valco) connected to a single-bead-string reactor (SBSR)[14]. The SBSR consisted of 80 cm of PTFE tubing filled with glass beads. The internal diameter of the tubing was 0.7 mm and the nominal diameter of the beads was 0.5 mm. The volume of this reactor was *ca.* 0.3 ml, providing a reaction time of 10–20 sec with the flow-rates used.

The biamperometric detector cell was of symmetrical thin-layer design and was made of KEL-F (Fig. 1B). It contained two 0.8-mm^2 platinum electrodes mounted in the same manner as detailed above and positioned directly opposite each other. A 100-μm thick PTFE spacer was used to separate the cell halves and a duct (3 × 6 mm) defined a cell volume of *ca.* 2 μl. The inlet and outlet to the cell were 0.15 mm I.D. PTFE tubings force-fitted through undersized through-bores in one of the cell halves and made flush with the interior surface. A high-pressure flangeless connector (Omnifit) was used to couple the cell inlet with the SBSR. The home-built potentiostat was of conventional design and equipped with a voltage output of ± 2 V and an offset function of ± 10 μA. The power supply was ± 15 V (Gardner Transformers) separate from the generator voltage supply above. The detector and generator-cell, the reactor and the mixing block were housed in a protective metal box. Normal earthing precautions were observed.

The detector current response was registered on an x–y recorder (BD 40; Kipp & Zonen) and all experiments were carried out at ambient temperature.

Chemicals and reagents

Mobile phases consisted of sodium phosphate buffer or monochloroacetic acid with ionic strength, pH and methanol content as noted in the text for the different applications. For the separation of aliphatic amines, sodium dodecyl sulphate (SDS) (Fluka, Buchs, Switzerland), was used as an ion-pairing agent. The reagent solution for bromine generation was prepared from the mobile phase by adding potassium bromide to a concentration of 0.1 *M* if not otherwise stated. Mobile phases were filtered through a 0.45-μm Millipore filter and degassed prior to use. Water from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) was used throughout. All chemicals were of p.a. grade.

Glutathione in the reduced form was 99% (Merck, Darmstadt, G.F.R.) and in the oxidized form 95% puriss. (Fluka). Methylamine was purum, 40% in water, ethylamine purum, 70% in water and propylamine purum. Lysozyme (egg white) and cytochrome *c* (horse heart) were from Sigma (St. Louis, MO, U.S.A.). Stock solutions were prepared and further diluted with mobile phase to the desired concentration.

RESULTS AND DISCUSSION

Consideration of detection parameters

Bromine generation. As indicated in the schematic representation of the detector system (Fig. 2), the reagent bromine is electrochemically generated from bromide by means of constant-current electrolysis in a separate flow. The amount of bromine produced can easily be adjusted by regulating the electrolysis current between 0.1 μA and 1 mA. This is an important feature since the indirect detection approach for monitoring the consumption of a reagent means that the optimum level of reagent must be a compromise between two conflicting demands: large linear dynamic range and low noise level. These two demands are further discussed below.

Constant-current efficiency for bromine generation was confirmed by the linear relationship between the generating current (I_g) and the corresponding amperometric response (I_d), see Fig. 3. The region of I_g shown, 5 to 200 μA, corresponds to bromine

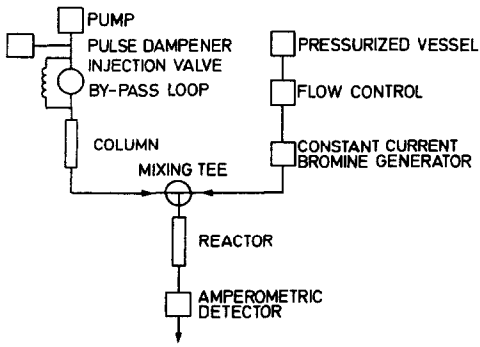


Fig. 2. Schematic representation of the reaction detection system developed.

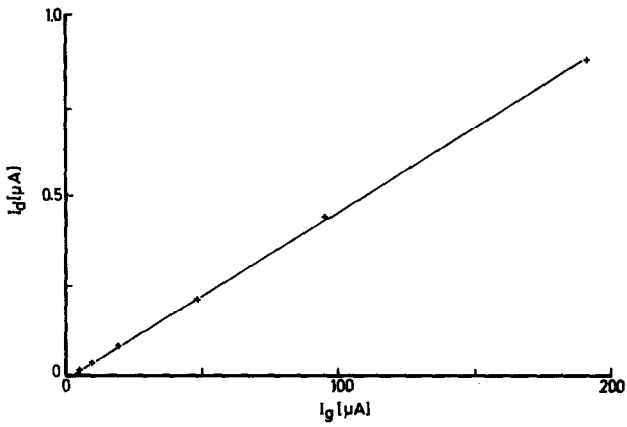


Fig. 3. Detector current (I_d) as a function of generator current (I_g). Mobile phase: 0.05 *M* sodium phosphate buffer, pH 4.5. Flow-rate, 1 ml/min. Reagent solution: mobile phase with 0.1 *M* potassium bromide. Reagent flow-rate, 0.5 ml/min. Calculated slope, 0.5%; intercept, −0.007 μA.

concentrations of 1 to 40 μ*M* in the combined column and reagent flow. Increasing I_g to 1 mA revealed a slight (less than 10%) decrease in current efficiency. This can be explained by the bromine concentration not being high enough. The small negative intercept visible in Fig. 3 is caused by bromine consuming impurities in the mobile phase. This intercept remained small as long as fresh buffer solutions and methanol of analytical grade were used. Consequently, the bromination pretreatment of the mobile phase utilized by King and Kissinger[12] was not found necessary.

The requirement of a medium inert towards bromine does not pose serious problems since a wide selection of suitable solvents and salts is available[15]. However, acetonitrile cannot be used since nitriles are susceptible to bromination in the α-position. Confirming the observations of King and Kissinger[12], no amperometric response was obtained with acetonitrile in the mobile phase despite a large generation of bromine.

Bromine reaction. The bromine reaction takes place during the passage of a reactor subsequent to the combination of the eluate and the reagent. A SBSR was chosen as it provides a more efficient micro mixing and a lower dispersion compared to an open-tubular reactor[14]. It still affords a low back-pressure, and a gas pressurized vessel (operable at pressures below *ca.* 8 bar) can be used to obtain a steady reagent flow. While reaction times of up to a few minutes should be possible with acceptable dispersion by using the SBSR[14], we have so far only used times of 10 to 20 sec.

Depending on reaction conditions, a linear dynamic range of one to three orders of magnitude will result. With our applications we found that up to 75% of the amount of bromine could be consumed before nonlinearity was apparent. Since the upper limit in this way is set by the amount of bromine available for reacton, the range can be extended by a larger generation of bromine. However, because of the amperometric monitoring technique used, the baseline noise will increase in proportion to the bromine concentration, and detection limits will hence be adversely affected.

Depending on the nature and number of groups that can be brominated in the

target analyte, the number of bromine molecules reacting with each analyte molecule can vary considerably and occasionally significant enhancement in sensitivity is realizable. This, in effect, chemical amplification will be illustrated in the application section.

Bromine detection. The baseline of the reaction detector consists of the amperometric response of bromine reduction when no bromine is consumed in the reactor. The concentration of bromine is continuously monitored and when reactive substances appear in the column effluent a decrease in the amperometric response will appear, giving the negative peaks in the chromatogram.

For bromine monitoring, a conventional three-electrode thin-layer cell[12,16] was experimentally compared with the more simple biamperometric two-electrode cell described in the experimental section. The latter cell was found preferable for reasons of simplicity, since the behaviour of the two cells was essentially the same. As working-electrode material, platinum was found preferable to glassy carbon due to its superior long-term stability. While a freshly polished glassy-carbon electrode displayed a sensitivity similar to that of a platinum electrode, this deteriorated rapidly and frequent repolishing of the glassy-carbon material was necessary. Platinum, on the other hand, could be used for extended periods of time (several months) without any pretreatment. The second platinum electrode in the two-electrode cell will, in the presence of bromide, serve as a quasi-reference having a potential or roughly 0.8 V *vs.* Ag/AgCl[17]. Hydrodynamic voltammograms were recorded and compared for the three-electrode cell and the two-electrode cell. The two voltammograms showed the same essential characteristics and agreed with the results reported by King and Kissinger[12]. For the two-electrode cell, a mass-transfer limited current was found in the region of -0.2 to -0.6 V *vs.* the quasi-reference electrode. Hence, a voltage of -0.4 V was chosen to be applied to the bimperometric detector cell. In this way the bromine monitoring will be highly selective and a certain precaution is realized against any occurrence of slower electrode kinetics caused by electrode passivation.

It was found essential not to switch the polarity of the two electrodes or otherwise very low and unstable bromine responses were encountered. A preliminary attempt to use a square-wave detection mode[18] failed, possibly because of the same reason.

Flow stability. Due to the inherent sensitivity of an amperometric response towards flow-rate variations[16,19,20], stringent requirements have to be put on the pumping system in order to obtain an adequate baseline stability. This is especially true in the present type of detection approach where a comparatively high concentration of electroactive compound is monitored.

For pumping the reagent, a gas pressurized vessel was found satisfactory with good flow-stability. The effect of different LC pumping systems is illustrated in Fig. 4. The comparison was made on the same occasion under identical conditions. A conventional high-quality dual-head reciprocating pump equipped with a pulse dampener produces large flow pulsations, as shown by trace a, while a positive displacement pump of the screw-driven syringe type shows much smaller pulsation (trace b). Among pulse-dampening devices, several conventional flexible diaphragm types were tested and found to give no effect in combination with the syringe pump (trace c), while the dampener based on an adjustable bellows-valve can reduce pulsations further (trace d). With the baseline stability optimized in this way, a change in I_d of

just 0.3% can be used for detection purposes. No improvement in baseline stability was achieved when bromine was generated directly in the column effluent.

The oscillations in baseline current, caused by flow pulsations from pressure surges induced at the injection, were reduced by inserting a by-pass loop in parallel with the injector[13].

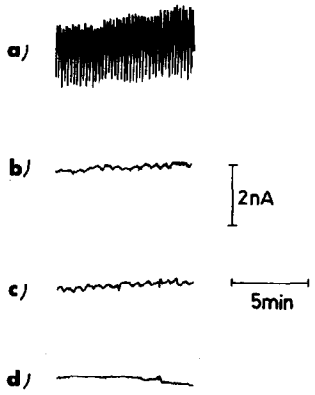
Selected applications

Glutathione. The disulphide and thiol forms of glutathione were chosen to illustrate how the presence of these common sulphur moieties render otherwise featureless molecular structures such as simple peptides detectable. According to Rauwel and Thevenot[21] the reactions with bromine proceed as

$$R\text{–}SH + 3Br_2 + 3H_2O \rightleftharpoons RSO_3H + 6HBr$$
$$R\text{–}SS\text{–}R + 5Br_2 + 6H_2O \rightleftharpoons 2RSO_3H + 10HBr$$

A certain amount of chemical amplification is thus realized under conditions of complete bromine reaction. For thiols, this is achieved by using an acidic medium while the disulphide reaction is enhanced at a neutral pH[21].

Fig. 5 shows a chromatogram recorded with the bromine reaction detector at low levels of the test analytes. The chromatographic separation was achieved by using a reversed-phase system as described by Allison and Shoup[22]. For the thiol form, its quantitative conversion can be confirmed by calculating the number of bromine equivalents experimentally consumed. This is calculated as the peak area (*i.e.*, the charge consumed) divided by the Faraday constant, the amount of analyte and the slope of I_d *vs.* I_g, making the assumption of 100% generating current efficiency. As evidenced in Fig. 5 the response for the disulphide form does not correspond to complete reaction with five bromine equivalents consumed since this would require a neutral medium. The chromatograms shown in Fig. 5 were recorded for a com-



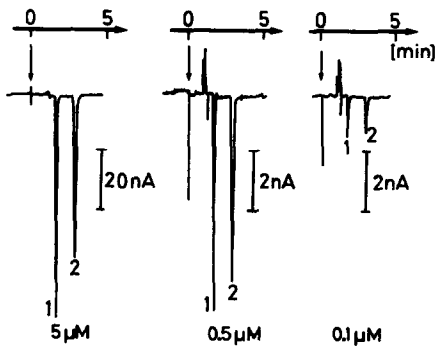


Fig. 4. Baselines with different combinations of LC pumps and pulse dampeners: (a) dual-head reciprocating pump including pulse dampener; (b) screw-driven syringe pump; (c) screw-driven syringe pump with a flow-through pulse dampener; (d) screw-driven syringe pump with a diaphragm type pulse dampener.

Fig. 5. Glutathione in reduced (1) and oxidized (2) form at concentrations 5 μM, 0.5 μM and 0.1 μM. Column: Spherisorb RP-18. Mobile phase: 0.1 *M* monochloroacetic acid (pH 3)–methanol (97:3). Flow-rate, 1 ml/min. Reagent solution: 0.1 *M* monochloroacetic acid (pH 3), 0.1 *M* potassium bromide. Reagent flow-rate, 0.6 ml/min. Generating current, 20 μA.

paratively low concentration of bromine (10 μM) in order to illustrate the detectability under conditions of a minimized baseline noise. For both the thiol and the disulphide form, a lower limit of detection of *ca.* 0.5 picomole injected can be estimated from Fig. 5 with a signal-to-noise ratio of 3. As a consequence of the low level of bromine, the linear dynamic range was limited to between 2 and 100 picomoles.

The repeatability of the detection system was tested with cysteine as the test substance. The thiol moiety in cysteine renders it readily detectable as discussed above. From 11 injections of a 10 μM solution a relative standard deviation of 1.6% was calculated. Similar observations have been made with the other compounds so far investigated. The expected reproducibility for LC analysis is thus realizable by using the bromine reaction detector.

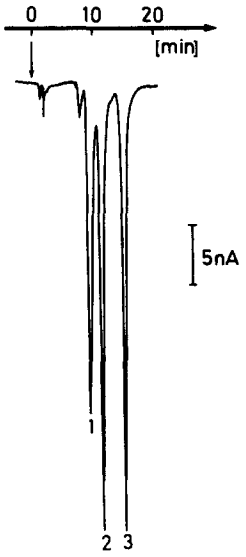
Aliphatic amines. Bromine can oxidize amino groups at a sufficient rate to make an analytical exploitation worthwhile. Coulometric titration procedures have long since been developed for ammonia[23]. Here, reactions were carried out in a weak alkaline medium in which the equilibrium of the bromine disproportionation reaction is such that the conversion into hypobromite and bromide is essentially quantitative. In a coulometric titration assay of amphetamine sulphate based on bromination of its primary amino group, however, Velghe and Claeys made use of a medium of pH 6.4[24].

Bromination rates for the bromine reaction detector are also adequate in the neutral pH range. This is illustrated in Fig. 6, which shows a chromatogram of methyl- ethyl- and propylamine recorded in a phosphate medium of pH 5.5. Compared to the experiment with glutathione, a longer reaction time proved necessary in this case, which was achieved by reducing the flow-rates by a factor of two. Obviously, the replacement of the reactor would equally well serve the same purpose. Response magnitudes indicate a bromine consumption of *ca.* 4 equiv., which is indicative of the expected formation of dibromoamines. This bromine consumption was not further increased when the pH was raised to 8.5, but decreased to a negligible extent at pH 4.8. The detection limits calculated with a signal-to-noise ratio of 3 were found to be *ca.* 10 picomoles.

The chromatographic separation of the aliphatic amines was achieved by a variation of the versatile dynamic ion-exchange chromatographic system developed by Kraak *et al.*[25]. In this case, SDS served as anionic ion-pairing agent. High concentration of SDS and bromide resulted in precipitation, and precautions to limit their concentrations were thus observed.

Proteins. Larger biomolecules, such as peptides and proteins, constitute an interesting group for bromine reaction detection. Here, opportunities in the same molecule for bromo-substitution reactions involving aromatic nuclei as well as bromine oxidation of, for example, thiol, disulphide and amino moieties (see above) together make a sizable chemical amplification possible[26]. A first example in this field is provided in Fig. 7, where the monitoring of lysozyme and cytochrome *c* after an isocratic ion-exchange separation is illustrated.

The bromination yields found correspond to 62 and 110 bromine equivalents for lysozyme and cytochrome *c*, respectively. Reducing the flow-rates by a factor of two, *i.e.*, doubling the reaction time, caused no improvement in bromine consumption. Neither did the use of a reaction medium of pH 8.5. The yields are about the same as were experimentally established with coulometric bromine titrations. In these



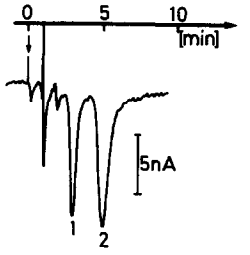


Fig. 6. (1) Methylamine (16 ng); (2) ethylamine (23 ng); (3) propylamine (33 ng). Column: Spherisorb RP-18. Mobile phase: 0.1 *M* sodium phosphate buffer (pH 4.5) with 0.2% SDS–methanol (65:35). Flow-rate, 0.5 ml/min. Reagent solution: 0.05 *M* sodium phosphate buffer (pH 7), 0.05 *M* potassium bromide–methanol (80:20). Reagent flow-rate, 0.35 ml/min. Generating current, 50 μA.

Fig. 7. (1) Cytochrome *c* (56 ng); (2) lysozyme (196 ng). Column: Mono S cation exchanger. Mobile phase: 0.05 *M* sodium phosphate buffer (pH 7), sodium bromide 0.3 *M*. Flow-rate, 1 ml/min. Reagent solution: same as mobile phase. Reagent flow-rate, 0.5 ml/min. Generating current, 30 μA.

titrations essentially the procedure detailed by Christian[27] was followed, with use of a constant current of 0.1 mA for bromine generation and an amperometric end-point detection. Thus, a short reaction time suffices for the sensitive detection of these two proteins.

Flow variations originating from the LC pump was in this case found to give a visible wave pattern in the baseline (Fig. 7). This was due to the exceptionally low operating pressure generated in the Mono S column (below 200 p.s.i. at a flow-rate of 1 ml/min), which resulted in a less efficient pulse dampening with the equipment available. Nevertheless, the detection limits calculated from the chromatogram shown (1.9 and 0.6 picomole for lysozyme and cytochrome *c*, respectively) represent the lower limit accessible with conventional UV detection.

CONCLUSIONS

The bromine reaction detector has been shown to be fully compatible with liquid chromatography when care is taken to avoid excessive band broadening. In this study, the reaction detector has been demonstrated to display a favourable sensitivity towards compounds such as aliphatic amines, thiols, disulphides and proteins. Its selectivity and the demonstrated detection limits in the low-picomole range imply that a number of problems in organic trace analysis can be addressed by using the developed detection approach.

Besides the inherent complexity, the most serious drawback of this type of detection is the stringent demands on flow stability, which require effective pulse-dampening devices for optimum performance.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. U. Forsman and B. L. Johansson for taking an active part in establishing the project and for many valuable discussions. Pharmacia AB (Uppsala, Sweden) is gratefully acknowledged for placing the Mono S columns at our disposal.

REFERENCES

1 P. T. Kissinger, K. Bratin, G. C. Davis and L. A. Pachla, *J. Chromatogr. Sci.*, 17 (1979) 137.
2 R. W. Frei, *Chromatographia*, 15 (1982) 161.
3 J. T. Stewart, *Trends Anal. Chem.*, 1 (1982) 170.
4 J. F. K. Huber, K.M. Jonker and H. Poppe, *Anal. Chem.*, 52 (1980) 2.
5 R. S. Deelder, A. T. J. M. Kuijpers and J. H. M. van den Berg, *J. Chromatogr.*, 255 (1983) 545.
6 R. W. Frei and A. H. M. T. Scholten, *J. Chromatogr. Sci.*, 17 (1979) 152.
7 C. J. Little, J. A. Whatley and A. D. Dale, *J. Chromatogr.*, 171 (1979) 63.
8 J. Crommen, B. Fransson and G. Schill, *J. Chromatogr.*, 142 (1977) 283.
9 H. Small and T. E. Miller, *Anal. Chem.*, 54 (1982) 462.
10 P. Jones, P. J. Hobbs and L. Ebdon, *Anal. Chim. Acta*, 149 (1983) 39.
11 C. E. Werkhoven-Goewie, W. N. A. Niessen, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 203 (1981) 165.
12 W. P. King and P. T. Kissinger, *Clin. Chem.*, 26 (1980) 1484.
13 J. L. DiCesare, M. W. Dong and J. R. Gant, *Chromatographia*, 15 (1982) 595.
14 J. M. Reijn, W. E. van der Linden and H. Poppe, *Anal. Chim. Acta*, 123 (1981) 229.
15 Z. E. Jolles (Editor), *Bromine and its Compounds*, Ernest Benn, London, 1966.
16 S. G. Weber and W. C. Purdy, *Ind. Eng. Chem., Prod. Res. Develop.*, 20 (1981) 593.
17 G. Nagy, Zs. Feher, K. Toth and E. Pungor, *Anal. Chim. Acta*, 100 (1978) 181.
18 B. G. Cox and W. Jedral, *J. Electroanal. Chem.*, 136 (1982) 93.
19 D. G. Swartzfager, *Anal. Chem.*, 48 (1976) 2189.
20 S. G. Weber, *J. Electroanal. Chem.*, 145 (1983) 1.
21 F. Rauwel and D. Thevenot, *Bioelectrochem. Bioenerg.*, 3 (1976) 284.
22 L. A. Allison and R. E. Shoup, *Anal. Chem.*, 55 (1983) 8.
23 G. M. Arcand and E. H. Swift, *Anal. Chem.*, 28 (1956) 440.
24 N. Velghe and A. Claeys, *Analyst (London)*, 98 (1973) 372.
25 J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.*, 142 (1977) 671.
26 W. J. Albery, R. Svanberg and P. Wood, *J. Electroanal. Chem.*, 162 (1984) 29.
27 G. D. Christian, *Anal. Biochem.*, 14 (1966) 183.