Journal of Chromatography, 462 (1989) 293–301
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 003

ION CHROMATOGRAPHY METHOD FOR IMINODIACETIC ACID DETERMINATION IN BIOLOGICAL MATRICES IN THE PRESENCE OF NITRILOTRIACETIC ACID

RENÉ P. SCHNEIDER, FRITZ ZÜRCHER, THOMAS EGLI* and GEOFFREY HAMER
Institute of Aquatic Sciences, Swiss Federal Institute of Technology (ETH) Zürich and Swiss Federal Institute for Water Resources and Water Pollution Control, Überlandstrasse 133, CH-8600 Dübendorf (Switzerland)

(First received July 29th, 1988; revised manuscript received September 27th, 1988)

SUMMARY

A sensitive ion chromatographic method for the determination of iminodiacetic acid in the presence of nitrilotriacetic acid in cell-free extracts is described using a mixture of carbonate, sodium hydroxide and 4-cyanophenol as the eluent. The eluent conductivity was chemically suppressed with a membrane suppressor and a conductivity detector was used for subsequent detection. The membrane was continuously regenerated with a sulphuric acid solution. Using a 20-μl injection loop, the detection limit for iminodiacetic acid was 230 μg/l. The influence of the eluent composition on the chromatography of iminodiacetic acid and interference by compounds present in biological matrices are discussed.

INTRODUCTION

The widespread eutrophication of lakes and rivers can be related to phosphate-containing discharges and run-off. A significant part of the phosphates in discharges from municipal sewage treatment works, which frequently are not equipped for effective phosphate elimination, is derived from household washing powders. In order to reduce phosphate loads in municipal sewage there is a trend towards replacement of the polyphosphates in washing powders by other chelating agents, *e.g.*, nitrilotriacetate (NTA). Hence, it has become essential to determine the fate of NTA in aqueous environments.

NTA has been shown to be biodegraded under both oxic and anoxic conditions[1–5]. The first enzyme involved in the metabolic pathway of NTA degradation in obligately aerobic bacteria was shown to be a monooxygenase which cleaves NTA into glyoxylate and iminodiacetic acid (IDA)[5–7]. However, the correct stoichiometry of this reaction has not yet been established and the metabolic fate of IDA still has to be elucidated[8].

Methods for the determination of NTA and glyoxylate in cell-free extracts are available[9,10]. Existing techniques for the determination of IDA have been evaluated

0021-9673/89/$03.50

and found to be ineffective for the problem cited above. The spectrophotometric method proposed by Bhattacharyya and Saha[11] was subject to interference by NTA, whilst gas chromatographic procedures[12,13] are both tedious and of poor reproducibility in cell-free extracts. Therefore, it became necessary to develop the new method described herein.

EXPERIMENTAL

Chemicals

All chemicals used were either of reagent grade or better and were obtained from either Fluka (Buchs/SG, Switzerland) or Merck (Darmstadt, F.R.G.).

Apparatus

The chromatographic system consisted of a Dionex DQP-1 pump, a Rheodyne 7125 injection valve fitted with an actuator position sensing switch (Rheodyne, Cotati, CA, U.S.A.), a 50-mm precolumn (Omnifit, Cambridge, U.K.) packed with a mixture of three parts of neutral precolumn material and two parts of macroporous cation-exchange resin (both from Sykam, Gauting, F.R.G.), an HPIC-AS3 ion chromatography column and an AMMS-1 anion micromembrane suppressor from Dionex (Sunnyvale, CA, U.S.A.). The eluent and regenerant were degassed under vacuum in collapsible containers (Cole-Palmer Instrument Company, Chicago, IL, U.S.A.). The regenerant was delivered to the suppressor with a minimicro 2/6 peristaltic pump (Ismatec, Zürich, Switzerland). With the exception of manufacturer fitted steel capillaries associated with the pump and the injection system and for Tygon tubing employed in the peristaltic pump, all connections were made of PTFE. The conductivity of the eluent was measured with a Sykam S3110 conductivity detector. The chromatogram was recorded on a W + W 600 recorder (W + W, Basle, Switzerland).

Reagents

Pure water was obtained from a NANOpure system (Barnstead, Boston, MA, U.S.A.). Eluent was delivered at 1 ml/min with a pressure of 4.4 MPa. A 0.05 *M* sulphuric acid solution was used as the regenerant at a flow-rate of 3.3 ml/min.

Solutions used for calibration

Pure water + IDA. Samples were prepared from a stock solution containing 30 mg/l IDA.

Tris–HCl + IDA. A 30 m*M* Tris solution containing 30 mg/l IDA was prepared and pH was adjusted to 7.5 with hydrochloric acid. Samples were obtained by appropriate dilution in 30 m*M* Tris–HCl pH 7.5.

Determination of IDA in cell-free extracts

The culture, harvest and disruption of cells from *Pseudomonas* sp. ATCC 29600 were performed as described by Schneider *et al.*[9]. Samples for calibration were prepared by diluting a cell-free extract stock solution containing 30 mg/l IDA in cell-free extract. Protein was precipitated by boiling of samples for 5 min. The precipitate was removed by centrifugation at 18 000 *g* for 5 min and the supernatant was used for IDA determination. Protein was determined by the method of Bradford[14].

Ion-exclusion chromatography (IEC)

IEC was performed as described by Schneider *et al.*[9].

RESULTS AND DISCUSSION

A primary problem in ion chromatographic analysis of IDA is its diprotic nature, *i.e.*, it changes its speciation with pH. This requires definition of the most appropriate of the three IDA species, $IDAH_2$, $IDAH^-$, IDA^{2-} (Fig. 1) for effective analysis.

Chromatography of $IDAH_2$

Under the acidic conditions (pH 2.8) prevalent in IEC, which was successfully employed for analysis of the structurally similar NTA[9], $IDAH_2$ is the predominant species. However,injection of this compound into the IEC system resulted in no peak, indicating a very strong affinity of $IDAH_2$ for the matrix material (IDA would have been detected as $IDAH^-$, as the pH of the eluent after passage through the suppressor was 7.0). Therefore, for analysis of IDA to be successful the pH of the eluent has to be increased to values where $IDAH^-$ or IDA^{2-} exist. This is the ideal operating range of standard ion chromatography, where IDA is detected as $IDAH^-$.

Chromatography of $IDAH^-$

First chromatography of the $IDAH^-$ species was evaluated using 0.5 m*M* bicarbonate, pH 8.3. Under these conditions no IDA peak was detected (Fig. 2, curve A). As only one carboxylic group is deprotonated at this pH, a large part of the $IDAH^-$ molecule containing the nitrogen-atom and the neutral carboxylic group might interact non-ionically with the resin matrix. This non-ionically adsorbed $IDAH^-$ might not be efficiently displaced by HCO_3^- and therefore no IDA peak was observed. Addition of 0.8 m*M* 4-cyanophenol to this eluent (pH maintained at 8.3) resulted in a broad, near symmetrical IDA peak (Fig. 2, curve B). This clearly demonstrates the ability of 4-cyanophenol to act as a modifier by reducing the column's capacity for non-ionic interaction. Even so, this column modification failed to give satisfactory chromatographic elution, suggesting that $IDAH^-$ is unsuitable for analysis.

Chromatography of IDA^{2-}

The alternative was to promote formation of the IDA^{2-} species by operation at even higher pH values. This was achieved by using different carbonate solutions as eluents. For an eluent containing 0.20 m*M* carbonate and 0.84 m*M* 4-cyanophenol (pH 9.5) a sharp peak with extensive tailing resulted (Fig. 2, curve C). In order to reduce the tailing, which was caused by the remaining $IDAH^-$, the carbonate concen-

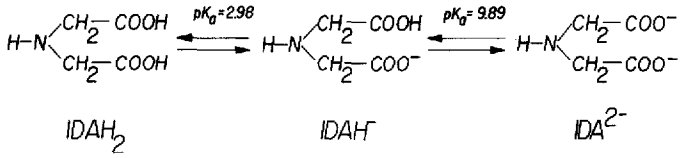

Fig. 1. Structures of the three possible IDA species and the respective pK_a values.

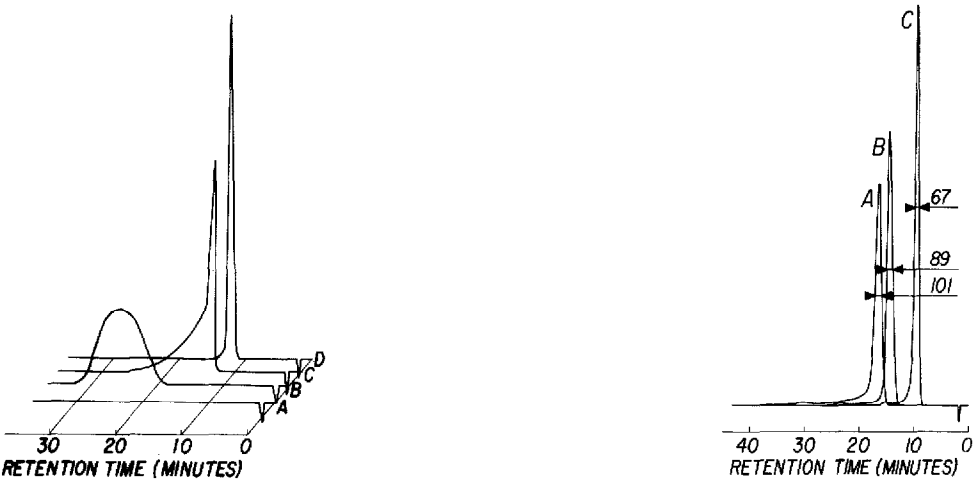

Fig. 2. Effect of different eluent compositions on chromatography of IDA: (A) 0.5 m*M* bicarbonate, pH 8.3; (B) 0.5 m*M* bicarbonate + 0.84 m*M* 4-cyanophenol, pH 8.3; (C) 0.2 m*M* carbonate + 0.84 m*M* 4-cyanophenol, pH 9.5; (D) 1.15 m*M* carbonate + 0.84 m*M* 4-cyanophenol, pH 10.2. A 20-μl volume of IDA standard solution (120 mg/l) was injected.

Fig. 3. Effect of different eluent compositions on chromatography of IDA^{2-}. Peak widths (in seconds) are given. (A) 0.98 m*M* carbonate, pH 10.7; (B) 1.16 m*M* carbonate + 2 m*M* sodium hydroxide, pH 10.90; (C) 1.11 m*M* carbonate + 2 m*M* sodium hydroxide + 0.84 m*M* 4-cyanophenol, pH 10.84. A 20-μl volume of IDA standard solution (120 mg/l) was injected.

tration in the above mentioned eluent was increased to 1.15 m*M* (resulting in pH 10.2). With this eluent, tailing was almost eliminated (Fig. 2, curve D), clearly indicating that for effective chromatography it is essential to convert most of the IDA into IDA^{2-}.

In order to comprehensively investigate the chromatographic behaviour of IDA^{2-}, further experiments were conducted with carbonate eluents (Fig. 3). With an eluent containing only carbonate (0.98 m*M*, pH 10.71) a broad peak (width 100.8 s) and slight tailing resulted (Fig. 3, curve A). The tailing was virtually eliminated by addition of 2.0 m*M* sodium hydroxide which increased the pH to 10.90 and also gave increased sensitivity (peak width 88.8 s; Fig. 3, curve B). Addition of 0.84 m*M* 4-cyanophenol to the carbonate–sodium hydroxide eluent resulted in a further improvement in sensitivity, the resulting peak width being 67.2 s with an eluent of pH 10.84 (Fig. 3, curve C).

From Figs. 2 and 3 it is clear that the eluent composition not only determines the peak shape but also affects the retention time. As there are four ionic species, *i.e.*, HCO_3^-, OH^-, CO_3^{2-} and deprotonated 4-cyanophenol (cnp^-) present in the eluent, experiments were conducted with controlled eluent compositions to investigate the impact of each ionic species on the residence time of IDA^{2-}. As the pH must be kept above 10.8 for efficient IDA chromatography (Fig. 3), changes in the species distribution in the HCO_3^-/CO_3^{2-} (pK_a 10.25) system have to be considered, whereas the speciation of cnp^- (pK_a 7.98) and OH^- are not affected at pH values above 10.8. In Fig. 4 the relationship between the retention time and the actual carbonate concentra-

tion is shown for a range of 4-cyanophenol concentrations in the eluent. The upper line represents pure carbonate, achieved either with carbonate alone or with added OH^-. The pH increase due to addition of OH^- will cause the HCO_3^-/CO_3^{2-} equilibrium to shift towards CO_3^{2-}. The points representing carbonate–sodium hydroxide eluents fit on the same line as points representing pure carbonate eluents (Fig. 4). Thus the carbonate concentration is the determining factor with respect to the retention time, whereas OH^- and HCO_3^- *per se* do not affect the elution strengths of the eluents. The remaining lines in Fig. 4 represent increasing 4-cyanophenol concentrations, 0.25, 0.50 and 0.84 m*M*, respectively, showing that 4-cyanophenol is, in addition to CO_3^{2-}, a retention time determinant.

It has already been shown that 4-cyanophenol functions as a modifier in the case of $IDAH^-$ (Fig. 2), but it is important to determine whether its effect is the same in the case of IDA^{2-}. In experiments where 4-cyanophenol–sodium hydroxide eluents (pH 10.8) were used, retention times for IDA^{2-} were inordinately long even at the highest 4-cyanophenol concentration tested, *i.e.*, > 50 min for 1.25 m*M* 4-cyanophenol, showing these combinations to be extremely weak eluents. The marked effect of 4-cyanophenol in carbonate eluents, as shown in Fig. 4, strongly suggests that 4-cyanophenol interaction with the column material also plays a rôle in the case of IDA^{2-}. As shown in Fig. 3, 4-cyanophenol addition to an eluent where most of the IDA is converted into IDA^{2-} does not dramatically affect the peak shape. This means that the mechanism of 4-cyanophenol action in chromatography of IDA^{2-} differs from its effect on analysis of $IDAH^-$. In the former case, 4-cyanophenol acts essentially as an accelerating agent, probably by blocking positively charged ion-exchange groups inside the resin, thereby leaving only the easily accessible cationic exchange groups on the resin surface to interact with IDA^{2-} and CO_3^{2-}, thus shortening diffusion pathways. Therefore, this mechanism should not be restricted to IDA^{2-}, as it is not based on a specific property of the IDA^{2-} molecule, but should

Fig. 4. Influence of carbonate and 4-cyanophenol on the retention time of IDA. The following eluent compositions are shown: ◇ = carbonate; ◆ = carbonate + 2 m*M* sodium hydroxide; ● = carbonate + 2 m*M* sodium hydroxide + 0.25 m*M* 4-cyanophenol; ▲ = carbonate + 2 m*M* sodium hydroxide + 0.50 m*M* 4-cyanophenol; ■ = carbonate + 2 m*M* sodium hydroxide + 0.84 m*M* 4-cyanophenol. Carbonate concentrations given in the figure are actual concentrations in the eluent. Lines connect eluents with constant 4-cyanophenol concentrations.

Fig. 5. Effect of 4-cyanophenol on the retention of IDA^{2-} (▲) and SO_4^{2-} (●). Dashed lines represent an eluent containing 0.37 m*M* carbonate + 2 m*M* sodium hydroxide; continuous lines represent an eluent containing 1.16 m*M* carbonate + 2 m*M* sodium hydroxide. Retention times are given relative to eluents containing no 4-cyanophenol.

also apply to other typical anions such as SO_4^{2-}. In Fig. 5 the effects of the 4-cyanophenol concentration on the retention time for both IDA^{2-} and SO_4^{2-} are shown for two carbonate eluents (0.37 m*M* carbonate + 2 m*M* sodium hydroxide; 1.16 m*M* carbonate + 2 m*M* sodium hydroxide). The similar behaviour of IDA^{2-} and SO_4^{2-} strongly supports the postulate that the mechanism of 4-cyanophenol action generally applies for anions and that IDA^{2-} behaves as a typical doubly charged anion.

Precipitation of proteins

For the analysis of IDA in cell-free extracts, the protein must be precipitated from the samples prior to chromatography. Protein precipitation with trichloroacetic acid (TCA) was successfully employed in the determination of NTA[9], but in the case of IDA this simple procedure was impossible because interference occurred between TCA and IDA during chromatography.

One possible solution to this problem is removal of TCA from samples prior to analysis. If the pH of the sample is adjusted to 1.8, most of the IDA present will be fully protonated ($IDAH_2$), whereas TCA will remain predominantly in the anionic state. As mentioned above, $IDAH_2$ is effectively adsorbed on resin material. Therefore, the use of a cationic exchange resin should allow efficient adsorption of $IDAH_2$, whereas negatively charged TCA anions should be excluded by the negatively charged resin. Acidified samples (pH 1.8 with hydrochloric acid) were applied to a 50-mm column (Omnifit) packed with Dowex 50W-X4 (20–50 mesh) (Fluka) which had been previously conditioned with hydrochloric acid, pH 1.8. The column was washed with five volumes of hydrochloric acid, pH 1.8. IDA was totally retained on the column and most of the TCA was removed in the waste. Elution of retained substances with an eluent containing 0.56 m*M* carbonate + 0.84 m*M* 4-cyanophenol + 2 m*M* sodium hydroxide was retarded due to initially complete protonation of the eluent ions by the excess of protons (H^+) which were loaded on the column during the conditioning and washing procedure. This caused the temporary build up of a pH gradient along the column. Desorption of IDA started only when most of the H^+ were neutralized such that the inlet composition of the eluent was progressively re-established. The pH gradient caused IDA to elute as a very broad peak, similar to that shown in Fig. 2 (curve B) but the residual TCA, which remained adsorbed on the resin after the washing procedure, still caused strong interference with IDA. The retardation effect and the pH gradient were almost eliminated by diluting the cationic exchange resin in neutral material, 98% styrene + 2% divinylbenzene copolymer (Fluka), but interference from TCA still remained prohibitive.

Another possibility for effective TCA removal is acidification to pH 0 with hydrochloric acid followed by extraction of the sample with diethyl ether[15]. IDA remains in the aqueous phase probably because of its positive charge due to protonation at the nitrogen atom. As diethyl ether is soluble in water at this low pH and as it decomposes the resin material, it had to be removed by extraction with tetrachloroethylene. The resulting solution could then be injected into the system without affecting its integrity. Although TCA had been quantitatively removed, no analysis of IDA was possible due to the high concentrations of inorganic anions introduced into the sample by acidification with hydrochloric acid.

Therefore, alternative methods for removing protein from the samples to be

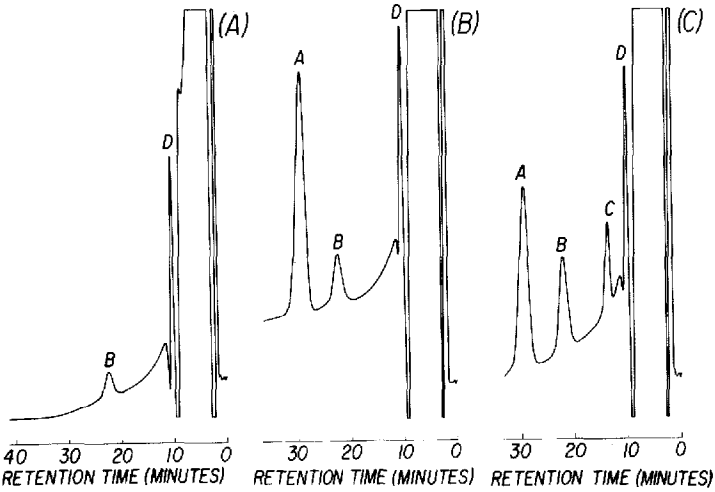

Fig. 6. Influence of matrix components on chromatography of IDA. (A) Tris–HCl buffer, 30 m*M*; (B) Tris–HCl buffer, 30 m*M*, containing cell-free extract; (C) same as (B) but containing 1 mg/l IDA. Protein concentration 3.0 mg/ml. Peaks: A = PO_4^{3-}; B = SO_4^{2-}; C = IDA^{2-}; D = unidentified compound. A 20-μl volume of sample was injected.

analysed were investigated. Filtration through protein-impermeable membranes (MPS-1; Amicon, Danvers, MA, U.S.A.) proved unsuccessful because of poor recovery of IDA in the filtrate. However, by boiling samples, protein could be denatured and precipitated such that it could be effectively removed by subsequent centrifugation with good recovery of IDA.

Analysis of cell-free extract

Final optimization of the eluent for use in the chromatographic analysis of IDA in cell-free extracts resulted in the following eluent composition: 0.56 m*M* carbonate + 0.84 m*M* 4-cyanophenol + 2 m*M* sodium hydroxide. In Fig. 6 three chromatograms are illustrated: (A) 30 m*M* Tris–HCl buffer; (B) cell-free extract in Tris–HCl buffer and (C) cell-free extract with IDA in Tris–HCl buffer. Several predominant peaks were associated with the Tris–HCl buffer employed, especially SO_4^{2-} (peak B) and an unidentified substance (peak D). The presence of 4-cyanophenol in the eluent was essential to move IDA (peak C) away from peak D. In the absence of 4-cyanophenol, peak D and IDA (peak C) overlapped irrespective of the carbonate concentrations employed.

Interferences

In the analysis of specific compounds in complex biological matrices, numerous materials that are generally present in variable concentrations frequently interfere with analytical procedures developed with artificial systems. To evaluate the extent of such potential interference, retention times for a wide range of possible interfering substances were determined in the system proposed (Table I). In general, interference was minimal, provided relatively low concentrations of the various compounds evaluated were present in the samples to be analysed.

TABLE I

RETENTION TIMES OF SOME POTENTIALLY INTERFERING COMPOUNDS COMMONLY FOUND IN BIOLOGICAL MATRICES

The concentrations tested were 200 mg/l and the injection volume was 20 μl. The eluent was 0.5 m*M* carbonate + 2.0 m*M* sodium hydroxide + 0.84 m*M* 4-cyanophenol.

Compound	*Retention time (min)*	*Compound*	*Retention time (min)*
Water	2.4	IDA	14.7
Propionate	2.4	Succinate	16.5
Acetate	2.4	Malonate	17.7
Butyrate	2.7	Maleate	18.9
Glycolate	3.0	NTA	22.0
Pyruvate	3.3	SO_4^{2-}	22.2
Cl^-	5.7	Oxalate	25.8
EDTA	8.4	PO_4^{3-}	28
NO_3^-	8.4	Fumarate	39
N-Methyl-IDA	12.3	Glycine	*
TCA	12.3	Sarcosine	–*
Malate	14.4		

* Compound not detected.

Calibration curves and sensitivity

In order to evaluate the efficacy of the proposed chromatographic method, analyses were performed for known concentrations of IDA in three different matrices: distilled water, 30 m*M* Tris–HCl buffer and cell-free extract. The results obtained are shown in Fig. 7. The calibration plots are parallel indicating no influence of the

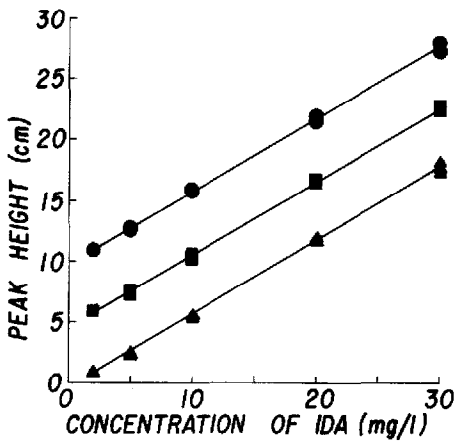

Fig. 7. Calibration plots obtained for IDA in different matrices. ● = IDA in pure water; ■ = IDA in Tris–HCl buffer, 30 m*M*; ▲ = IDA in cell-free extract (protein concentration in 300 m*M* Tris–HCl buffer, pH 7.5, was 30 mg/ml). To clearly distinguish between the different calibration plots shown in the figure, 5 cm and 10 cm were added to peak height values obtained from IDA in 30 m*M* Tris–HCl, pH 7.5 and for IDA in pure water, respectively. Three experiments were performed for each concentration tested. Calibration plots were obtained from linear regression analysis. A 20-μl volume of sample was injected.

matrix on the peak response. Replicate analyses were reproducible to within 2.8%. The calculated limit of determination[16] using a 20-μl injection loop was 230 μg IDA per litre for samples containing cell-free extract; it was considerably below 50 μg/l in distilled water, but no attempts were made to extensively characterize the system performance in this range. As the eluent pH has to be kept above 10.8 and interferences by other compounds in chromatography of IDA have to be avoided, very little flexibility with respect to modification in eluent composition exists. Such minor possible modification will not affect the sensitivity because a very small change in the ionic composition of the eluent will cause only a slight shift in eluent pH after suppressor passage such that neither background conductivity nor IDA protonation will change considerably. The sensitivity for determination of IDA in cell-free extract can be improved by utilizing off-line concentration columns of the type used for removal of TCA. If samples with low concentrations of IDA are to be analysed it is indispensable to use detectors equipped with well designed thermostated conductivity cells. In our experience, electronic temperature compensation of poorly (thermally) insulated detector cells produced unacceptable noise levels when high sensitivity measurements were needed.

ACKNOWLEDGEMENTS

R. P. S. thanks the Research Commission of ETH Zürich for financial support. Our thanks are due to Walter Suter, Hans-Ulrich Weilenmann and Mario Snozzi for technical and scientific support and to Mr. P. Schlup for photographic work.

REFERENCES

1 L. Anderson, E. Bishop and L. Campbell, *CRC Crit. Rev. Toxicol.*, 15 (1985) 1.
2 T. Egli, H. U. Weilenmann, T. El-Banna and G. Auling, *System. Appl. Microbiol.*, 10 (1988) 297.
3 S. O. Enfors and N. Molin, *Water Res.*, 7 (1973) 881.
4 T. Egli and H. U. Weilenmann, *Experientia*, 42 (1986) 1061.
5 M. K. Firestone and J. M. Tiedje, *Appl. Environ. Microbiol.*, 35 (1978) 955.
6 R. E. Cripps and A. S. Noble, *Biochem. J.*, 136 (1973) 1059.
7 M. K. Firestone, S. D. Aust and M. Tiedje, *Arch. Biochem. Biophys.*, 190 (1978) 617.
8 T. Egli, *Microbiol. Sci.*, 5 (1988) 36.
9 R. P. Schneider, F. Zürcher, T. Egli and G. Hamer, *Anal. Biochem.*, 173 (1988) 278.
10 F. Trijbels and G. D. Vogels, *Biochim. Biophys. Acta*, 113 (1966) 292.
11 S. N. Bhattacharyya and N. C. Saha, *Talanta*, 23 (1976) 331.
12 C. B. Warren and E. J. Malec, *J. Chromatogr.*, 64 (1972) 219.
13 R. A. Larson, J. C. Weston and S. M. Howell, *J. Chromatogr.*, 111 (1975) 43.
14 M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
15 R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, *Biochemical Databook*, Clarendon Press, Oxford, 3rd ed., 1986.
16 W. Funk, V. Dammann, C. Vonderheid and G. Oehlmann, *Statistische Methoden in der Wasseranalytik*, VCH, Weinheim, 1st ed., 1985.