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ENRICHMENT OF COMPLEXING ANALYTES ON A COPPER-LOADED SILICA SURFACE AND ON-LINE COUPLING WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, USING L-TRYPTOPHAN AND L-TYROSINE AS MODEL COMPOUNDS

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SUMMARY

A chemically modified silica (bisdithiocarbamate) strongly binds copper and has a loading capacity of 0.5 mmol g^{-1} for the copper ion. This new metal-loaded silica, packed in a short stainless-steel pre-column, is highly selective for the enrichment of two amino acids chosen as model compounds. On-line coupling of this pre-column with an analytical RP-18 column allowed the separation and detection of L-tryptophan and L-tyrosine at parts per billion levels in aqueous media with good accuracy. Separation was achieved by ion-pair chromatography and the solutes were detected by fluorescence. The ions generally present in natural aqueous media did not interfere.

INTRODUCTION

The determination of trace components in complex samples by high-performance liquid chromatography (HPLC) cannot be carried out directly, an initial pre-treatment of the sample being necessary in order to enrich the solute of interest and to reduce interferences. Natural waters containing, for example, amino acids at parts per billion levels require pre-concentration prior to analysis. Freezing concentration, among other methods has been used as an off-line pre-concentration technique for these species[1].

The possibility of using appropriate solid phases in a short stainless-steel pre-column for this purpose has been extensively studied[2–4]. Further, this pre-column can be coupled on-line with an analytical column. Many different phases have already been used, including styrene–divinylbenzene copolymers, C_{18}- or C_8-modified silica and activated carbon, but all have been found to be non-selective as the retention of solutes on these surfaces is non-specific (adsorption, dispersion, etc.).

For polar compounds, pre-concentration based solely on hydrophobicity is not sufficient and it is therefore necessary to use a surface that exhibits greater selectivity with respect to compound retention. Compounds with heteroatoms that possess a free electron pair show electron-donating properties (*i.e.*, Lewis bases) and

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can act as complexing ligands towards transition metals bound to a solid phase. This property is used extensively in ligand-exchange chromatography (LEC)[5].

Organic analytes that possess complexing substituent groups can be efficiently pre-concentrated on metal-loaded stationary phases. Examples given in the literature include the following:

(1) A copper-loaded iminodiacetate-modified silica (IDA) has been used for the pre-concentration of catecholamines. The pre-column was subsequently coupled on-line with a C_8 silica column for the separation of these compounds[6].

(2) A platinum-loaded 2-amino-1-cyclopentene-1-dithiocarboxylic acid-modified silica was shown to be able to act as an aniline filter for a group separation of herbicides from the aniline[7].

(3) A mercury–8-hydroxyquinoline phase has been used for the selective enrichment of 2-mercaptobenzimidazole. Following this procedure, the pre-column was coupled on-line with a C_{18} silica column[8].

(4) Our group has recently developed two copper-loaded silicas for the pre-concentration of L-tryptophan and carboxylic acids in aqueous media[9]. A bisdithiocarbamate silica surface was chosen as a chelating support owing to its strong binding with transition metals.

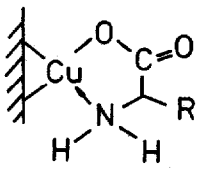
The mechanism of the retention of amino acids on metal-loaded silica[10] was determined by employing a bisdithiocarbamate silica loaded with copper (capacity 0.5 mmol g^{-1} of Cu^{2+}). The most important interaction was found to be complexation between the copper ion and the amino acid functional group, the species formed being a metal chelate complex with a five-membered ring at pH >5 (Fig. 1).

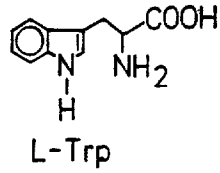
The aim of this work was to study the coupling of the enrichment procedure with a chromatographic separation of two amino acids, L-tryptophan and L-tyrosine (L-Trp and L-Tyr), as model compounds. The structures of the two amino acids are shown in the Fig. 2. This coupling allow us to determine these two amino acids directly in aqueous media by HPLC.

EXPERIMENTAL

Apparatus

Chromatographic studies were performed with an LC-5000 liquid chromatograph (Varian, Palo, Alto, CA, U.S.A.). A Model 414-T pump (Kontron, Zürich, Switzerland) was used for the enrichment step. These two pumps were used in combination with Models 7000 and 7125 six-port switching valves (Rheodyne, Berkeley, CA, U.S.A.), A Varian Fluorichrom fluorimetric detector operated at excitation wavelength 280 nm and emission wavelength 340 nm and a Model 1100 recorder (W+W, Basle, Switzerland).





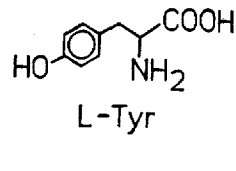


Fig. 1. Formation of the copper–amino acid complex.

Fig. 2. Structures of L-Trp and L-Tyr.

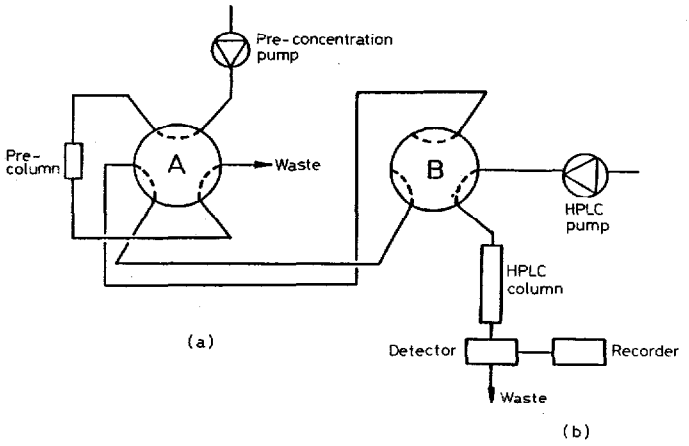


Fig. 3. On-line coupling of the enrichment step with the chromatographic separation. A and B = six-port switching valves. (a) Enrichment step; (b) elution and chromatographic separation.

Reagents and solvents

The amino acids were obtained from Merck (Darmstadt, F.R.G.). HPLC-grade methanol was obtained from Romil (Shepshed, U.K.). All other compounds were purchased from Fluka (Buchs, Switzerland) and all aqueous solutions were prepared using doubly distilled, deionized water.

The eluents were filtered and degassed in a Millipore (Bedford, MA, U.S.A.) filtration system before the chromatographic analyses.

Preparation of copper-loaded bisdithiocarbamate silica (bis-DTC-Cu)

The covalent binding of the bisdithiocarbamate (bis-DTC) to 10 μm Polygosil silica gel (Macherey-Nagel, Düren, F.R.G.) was carried out as described previously[9,10]. The copper loading was performed by mixing 1 g of silica bis-DTC with 100 ml of an acetate buffer solution (pH 5.6) containing 10% of 0.1 *M* copper(II) chloride. The mixture was stirred for 30 min at room temperature. Finally, the silica was filtered, washed with doubly distilled water and dried under vacuum.

The amount of copper not retained was determined by EDTA titration of the filtrate with murexide as indicator. This result allowed the copper capacity of the surface to be calculated (0.5 mmol g^{-1}).

Procedure

Pre-concentration and HPLC analysis were carried out using the coupling shown schematically in fig. 3.

The 10 × 2 mm I.D. pre-column (laboratory fabricated) was slurry packed with a syringe using an aqueous slurry of 10 μm bis-DTC-Cu. The separations were carried out using a 150 × 4.6 mm I.D. column packed with 5-μm RP-18 Micropack MCH-5 silica (Varian).

Stock solutions of 100 ppm of L-Trp and L-Tyr in doubly distilled, deionized water were stored in the dark at 5°C. Standard solutions (1–250 ppb)* were prepared

* Throughout this article, the American billion (10^9) is meant.

daily and filtered on a 0.22-μm membrane filter. Before the enrichment procedure, the bis-DTC-Cu silica packed in the pre-column was washed with 30 ml of water at 1 ml min^{-1}, after which 10–20 ml of the amino acid solutions were percolated at 1 ml min^{-1} through the pre-column. Following this enrichment step, the valves were switched in order to couple the pre-column on-line with the analytical column as shown in Fig. 3. The acidic mobile phase (pH 2.25) pumped at a flow-rate of 1 ml min^{-1} eluted the amino acids from the pre-column. These compounds were then separated by ion-pair chromatography on the RP-18 column and detected by fluorescence (excitation wavelength 280 nm, emission wavelength 340 nm).

RESULTS AND DISCUSSION

The breakthrough volumes (V_B) were determined using 200–500 ppb aqueous solutions of L-Trp and L-Tyr. The influences of pH and ionic strength on the breakthrough volume for L-Trp have already been discussed[9]. The same results were obtained for L-Tyr, *i.e.*, the breakthrough volume was maximal at pH >5 and ionic strength had no influence on the retention of amino acids by the copper-loaded silica.

The breakthrough volumes determined with the 10 × 2 mm I.D. pre-column at pH 6.5 in 0.1 *M* sodium perchlorate were 27 and 14 ml for L-Trp and L-Tyr, respectively. The difference observed between the two breakthrough volumes was attributed to the participation of the indole group of L-Trp with the copper[11], which serves to supplement the principal five-membered ring mechanism determined previously[10].

Chromatography of L-*Trp*

Following the enrichment step, the amino acid was separated on a C_{18} analytical column by ion-pair chromatography. The chromatograms obtained for different aqueous solutions are shown in Fig. 4. The linearity of the graph of peak area *versus* the amount of L-Trp pre-concentrated ($r = 0.9952$) demonstrates that the amino acid was quantitatively retained by the pre-column in this range (0–600 ng).

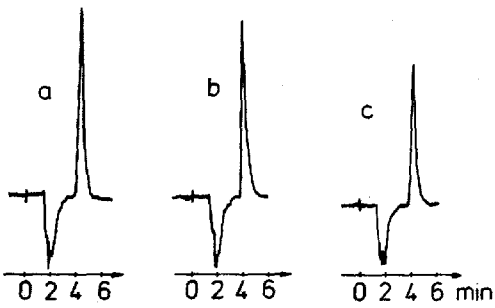
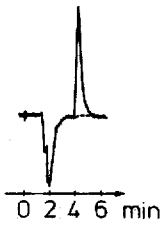


Fig. 4. Chromatograms of aqueous L-Trp samples. (a) 20 ml of 30 ppb L-Trp in 0.1 *M* sodium perchlorate; (b) 20 ml of 30 ppb L-Trp in 0.1 *M* sodium perchlorate + $8 \cdot 10^{-3}$ *M* calcium chloride; (c) 10 ml of 50 ppb L-Trp in 1 *M* sodium chloride. Chromatography on a 150 × 4.6 mm I.D. column packed with 5-μm Micropack MCH-5. Mobile phase, 50% methanol in 0.04 *M* disodium hydrogen citrate buffer containing $4 \cdot 10^{-4}$ *M* dodecyl sodium sulphate. This mobile phase was adjusted to pH 2.25 by adding 2 *M* hydrochloric acid[12]. Flow-rate, 1 ml min^{-1}. Fluorescence detection: excitation wavelength 280 nm and emission wavelength 340 nm.



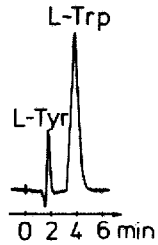


Fig. 5. Chromatogram of 10-ml river samples without (broken line) and spiked with 50 ppb of L-Trp (solid line). Enrichment using chromatographic conditions as in Fig. 4.

Fig. 6. On-line pre-concentration of 10 ml of an aqueous solution containing 102 ppb of L-Trp and 255 ppb of L-Tyr. Analytical conditions as in Fig. 4.

A recovery study was conducted by comparison of the peak areas obtained for equivalent amounts of L-Trp after enrichment and on direct injection. The recoveries obtained for 0.1, 0.2 and 1 μg of L-Trp were higher than 95%.

The reproducibility of the procedure was calculated from nine replicate determinations on directly injected and enriched samples that contained 1 μg of L-Trp. The average recovery was 105% with a coefficient of variation of 4.8%.

These results demonstrate that coupling between the pre-column and the analytical column was very suitable for these amino acid analyses. The detection limit determined for L-Trp in aqueous solution was lower than 1 ppb.

Application to natural samples

Some alkali and alkaline-earth metals are always present in natural waters and these species could conceivably interfere with amino acid analyses. To study this effect we pre-concentrated L-Trp aqueous solutions containing varying concentrations of sodium chloride (0–1 *M*) and calcium chloride (0–8 · 10^{-3} *M*). The chromatograms obtained for these solutions, when compared with those obtained without the salts, demonstrate that no interference occurred (Fig. 4).

A water sample collected from the Arve River (Geneva) and filtered through a 0.22-μm filter was spiked with 50 ppb of L-Trp. The results for the blank (unspiked sample) showed the absence of the amino acid at the detection limit. Fig. 5 is the chromatogram obtained for the blank and for the river sample spiked with 50 ppb of L-Trp. The efficiency of the method is clearly demonstrated by comparing the chromatograms obtained for real and synthetic samples.

Chromatographic separation of L-Trp and L-Tyr

The linearity, recovery and repeatability of the L-Tyr analyses were also studied. The same results were obtained as for L-Trp, except for the detection limit, because the wavelength used for fluorescence detection was maximal for L-Trp but not for L-Tyr, resulting in a detection limit for L-Tyr under these conditions of only 20 ppb.

A chromatogram of L-Trp and L-Tyr obtained after on-line enrichment of a 10-ml sample is shown in Fig. 6, and indicates a satisfactory resolution, R_s = 1.6. Compared with a 50-μl loop injection, the recovery was found to be 100%.

Re-usability of the copper phase

The bis-DTC-Cu exhibits good stability in the pH range 5–8[13]. Below this pH, the copper–dithiocarbamate complex is not stable, as mentioned elsewhere[14]. Elution of the two amino acids with an acidic mobile phase (pH 2.25) would therefore destroy the amino acid–copper complex and the metal-loaded silica. For this reason, we do not re-use this phase.

Considering the low cost of this procedure (only 40 mg of the copper phase is used) and the ease of re-packing the pre-column, the lack of re-usability of this copper phase is considered to be only a minor inconvenience.

CONCLUSIONS

The bis-DTC-Cu has been shown to be an effective means for the selective pre-concentration of amino acids in aqueous media. These compounds were retained on the metal-loaded surface by formation of a chelate complex at pH > 5.

The possibility of on-line connection of the enrichment pre-column packed with the metal-loaded surface to an RP-18 analytical column has been demonstrated for two model amino acids, L-Trp and L-Tyr. This coupling allowed a natural sample to be analysed directly by ion-pair chromatography. The ions generally present in aqueous samples did not interfere.

The detection of amino acids at parts per billion levels was possible with excellent reproducibility and a recovery of nearly 100%. This procedure can, therefore, be considered to be applicable to the determination of amino acids in aqueous media.

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