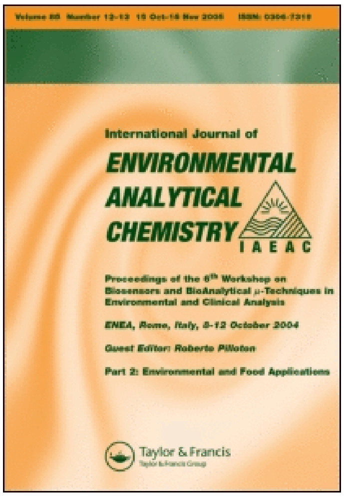

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Enrichment of Amino and Carboxylic Acids Using Copper-loaded Silica Pre-columns Coupled On-line with HPLC

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A novel method for preconcentrating amino and carboxylic acids on silica surfaces has been described. Two different copper-loaded silica surfaces, bis-dithiocarbamate-copper silica (Bdtc-Cu-Si) and cyclam-copper silica (Cy-Cu-Si) were prepared for this purpose. Bdtc-Cu-Si proved to be selective for amino acids, whereas, Cy-Cu-Si proved to be selective for carboxylic acids. These newly prepared surfaces showed good stability and strong binding properties toward organic compounds. Inorganic anions did not seem to show interference with amino acids, whereas, an excess of these ions interfere with the carboxylic acid pre-concentration.

These surfaces were packed in pre-columns and then were coupled on-line with HPLC. Nanogram quantities of the organic compounds of interest may be determined using this technique.

KEY WORDS: Sample handling, trace analysis, copper-loaded silica.

INTRODUCTION

The analysis of trace components in complex samples by HPLC cannot be done directly: an initial enrichment step is necessary. A new approach consists in on-line coupling of a pre-column packed with a suitable sorbent, followed by an analytical column.[1]

Sorbents, such as silica, alumina, porous polymers or modified silica are contained in short stainless steel or glass columns. Nevertheless, these sorbents are often unselective and can sometimes even retain undesirable compounds. Hence, our goal was to develop a more selective sorbent surface.

It is well known that anions or neutral molecules possessing a free electron pair show electron-donating properties (Lewis bases) and act as complexing ligands toward transition metals. The most common ligands are: amines, carboxylic acids, alcohols, mercaptans, sulfides, as well as compounds having several functional groups such as hydroxy-acids, hydroxy-amines and amino acids.

Amino and carboxylic acids are known to form labile complexes with copper(II). Hence, we developed some new copper-loaded silica surfaces for the selective enrichment of these compounds.

EXPERIMENTAL

1. Preparation of copper-bonded-silica surfaces

We prepared two chelating surfaces such that the copper would be strongly bound to the silica surface. This is a pre-requisite for the enrichment step because under these conditions it is necessary that the copper be irreversibly bound. The description of their preparation is given below:

a) Bis-dithiocarbamate-copper-silica surface (Bdtc-Cu-Si)

Silica gel (Polygosil 60-10, particle size 10 μm) was "silanized" with N-β-amino-ethyl-amino-propyl-trimethoxy-silane (Dow Corning Z-6020). Then the amino groups were converted into dithiocarbamates and finally the chelated silica was loaded with copper as shown in Figure 1.

The loading capacity of the prepared surface was 0.5 mmole Cu/g of silica.

b) Synthesis of Cyclam-copper-silica surface (Cy-Cu-Si)

The first step consisted in attaching 3-glycidyloxypropyl-trimethoxysilane (Fluka AG, Buchs) to the silica surface (Polygosil 60-4063, particle size 40 to 63 μm). Then, the resulting product was reacted

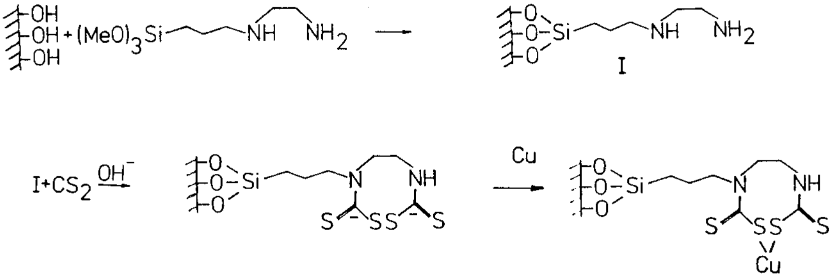


FIGURE 1 General reaction scheme for the preparation of Bdtc-Cu-Si.

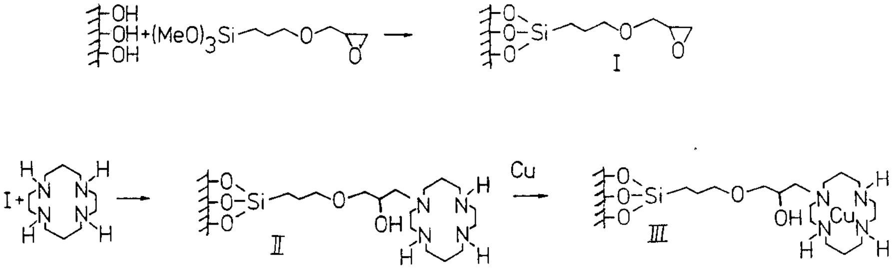


FIGURE 2 General reaction scheme for the preparation of Cy-Cu-Si.

with cyclam in chloroform medium for about 60 hours. The cyclam-silica surface was then loaded with copper as shown in Figure 2.

It should be noted that in structure III more than one hydrogen may be substituted on the nitrogen atom. We are still studying this aspect. The loading capacity of this surface was about 100 μmoles Cu/g of silica, which is lower than that of the Bdtc-Cu-Si surface but is, nevertheless, sufficient for enrichment purposes.

2. Enrichment and on-line elution

The apparatus used for this purpose, is shown schematically in Figure 3.

a) Enrichment step

Teh to 20 ml of the test sample were pumped through the pre-column using a flow rate of 1 ml/min (Pump a in Figure 1).

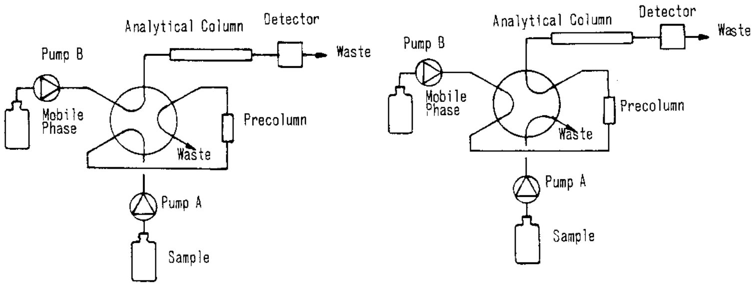


FIGURE 3 Enrichment and separation steps: Pump A, Kontron LC Pump 414-T; Pump B, Varian 5000 Liquid Chromatograph; Switching valve (SV), Rheodyne 7010; Analytical column, RP-18, Micropack MCH-5 Varian 15 cm × 4.6 mm I.D.; Pre-column, Home-made pre-column 10 × 2 mm I.D.; Detectors, UV-detector Varian UV-100 and fluorescence detector Varian Fluorichrom; Mobile phases, Specified in Figures 7 and 8.

b) Separation step

After the enrichment step, the pre-column was coupled on-line via a six-port switching valve to an analytical column mounted on an HPLC system.

3. Possible applications of chelated silica surfaces

To illustrate the application of these chelated surfaces, we chose the following compounds for enrichment separation:

a) Amino acids on Bdtc-Cu-Si:

L-Tryptophan (L-Trp) (Merck AG, Darmstadt) was chosen as the model compound for our analysis since it can be detected by fluorescence as shown in Table I.

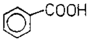
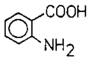
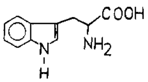
b) Carboxylic acids on Cy-Cu-Si:

As for the amino acids, model compounds such as benzoic and anthranilic acids (Fluka AG, Buchs) were chosen for our study (Table I).

The pre-column that we used[2] was packed with the appropriate chelated surfaces.

TABLE I

UV-absorbance maxima and pKa's of carboxylic acids. Fluorescence properties and pKa's of L-Trp

Name	Formula	Absorbance	pKa
Benzoic acid		λmax = 227 nm	4.19
Anthranilic acid		λmax = 247 nm λmax = 332 nm	2.10 4.94
Name	Formula	Fluorescence	pKa
L-Trp		λex = 287 nm λem = 348 nm	2.35 9.39

RESULTS AND DISCUSSION

1. Analysis of amino acids

Various parameters such as breakthrough volume, the effects of pH and ionic strength and optimal conditions for the elution of amino acids were studied.

a) Determination of the breakthrough volume

The breakthrough volume, V_b, was determined with a 500 ppb L-Trp aqueous solution. An average breakthrough volume value of 30 ± 3 ml was obtained from six measurements. Similar results were obtained using lower L-Trp concentrations.

b) Effect of pH

The influence of the pH on the breakthrough volume was studied at constant ionic strength (0.1 M $NaClO_4$). The pH was adjusted either with 0.1 M $HClO_4$ or 0.1 M NaOH. The results are shown in Figure 4.

It can be seen that the active species which binds to the metal is the anionic form (L-Trp$^-$) of the amino acid (Figure 5). This observation is consistent with the results cited in the literature.[3]

c) Effect of ionic strength

The influence of ionic strength was studied by keeping the pH

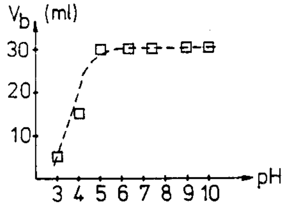
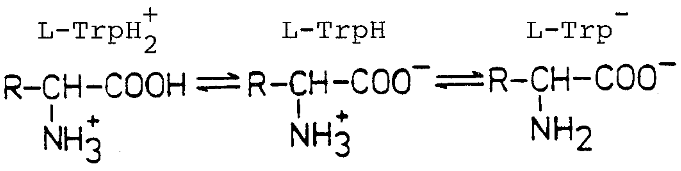


FIGURE 4 Breakthrough volume, V_b, versus the pH of the mobile phase.



$$\overset{\text{L-TrpH}_2^+}{\mathrm{R{-}\underset{NH_3^+}{CH}{-}COOH}} \rightleftharpoons \overset{\text{L-TrpH}}{\mathrm{R{-}\underset{NH_3^+}{CH}{-}COO^-}} \rightleftharpoons \overset{\text{L-Trp}^-}{\mathrm{R{-}\underset{NH_2}{CH}{-}COO^-}}$$

FIGURE 5 Acid-base equilibrium.

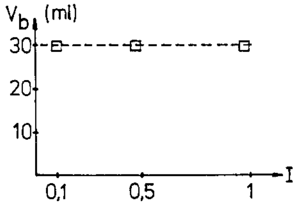


FIGURE 6 Breakthrough volume, V_b, versus ionic strength.

constant at 6.5. The ionic strength was varied between 0.1 and 1 M using $NaClO_4$ as the electrolyte. The results are shown in Figure 6 and it can be seen that V_b is unaffected by the ionic strength under these conditions.

d) Elution of amino acids

Since the enriched test compound is retained by the copper in its anionic form, it must first be protonated to elute it from the precolumn. An acidic mobile phase has been used to elute amino acids in ion-pairing chromatography.[4]

We compared the results from a loop injection of 200 ng of L-Trp on 20 μl sample with that of the same quantity in a 20 ml sample i.e.

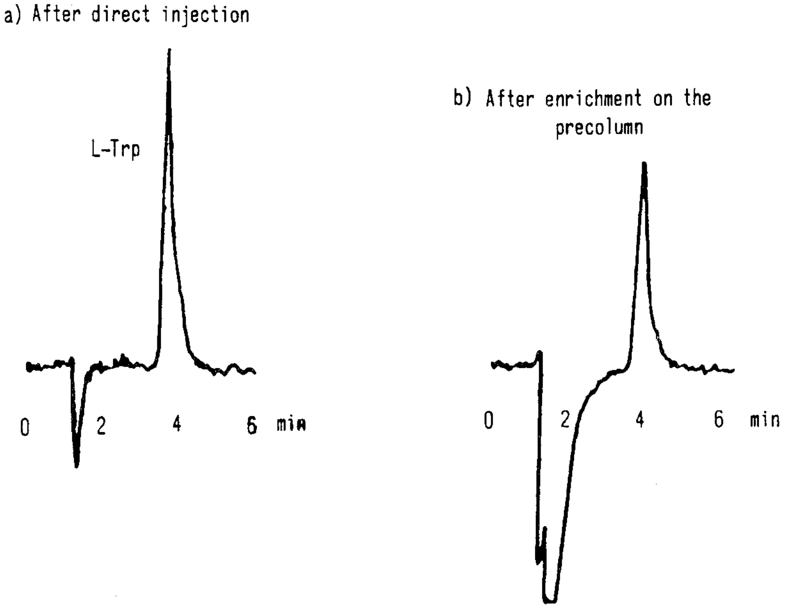


FIGURE 7 (a) Chromatogram of a direct injection of L-Trp (200 ng in 20 μl). (b) Chromatogram of a preconcentrated sample of L-Trp (200 ng in 20 ml). Fluorescence detection (Varian Fluorichrom) λex = 280 nm and λem = 340 nm; Flow rate 1 ml/min; Mobile phase, 50% MeOH (HPLC grade Romil Chem.) in 0.04 M di-sodium hydrogen citrate buffer containing 4×10^{-4} M dodecyl sodium sulfate (Fluka AG, Buchs). This mobile phase is adjusted to a pH of 2.25 by adding 2 N HCl[4].

1000 times more dilute, which was first preconcentrated on the precolumn and then injected for analysis. Results, as shown in Figure 7, reveal that recovery of the preconcentrated sample was approximately 80%. Losses may be due to incomplete elution of the L-Trp from the pre-column. Improved recoveries may be obtained by lowering the pH of the mobile phase at the expense of detector sensitivity loss. Therefore, as a compromise, a pH of 2.25 was chosen for elution purposes.

2. Analysis of carboxylic acids

The influence of different physical–chemical parameters such as breakthrough volume, interference effects and optimal conditions for elution were studied.

a) Breakthrough volume

The breakthrough volume was determined using a solution of 1 ppm anthranilic acid. It was found to be 10 ± 2 ml. Up to the breakthrough volume, normally proportionality between the preconcentration volume and the elution peak is respected, whereas, this is no longer true beyond the breakthrough volume. However, in our case, we observed that proportionality was respected for a preconcentration volume which was twice that of the breakthrough volume. At present the cause for this observation is not clear.

b) Preconcentration mechanism

To understand the mechanism by which the preconcentration occurs, various organic compounds dissolved in bi-distilled water were tested. At this pH, 7, the carboxylic test compounds are found primarily in their carboxylate form. In this form, they were found to be efficiently preconcentrated. Under the same experimental conditions, *p*-toluene sulfonic acid also found in its anionic form, yielded similar results. However, neutral molecules such as aniline, pyridine and phenol could not be preconcentrated. These results indicate that the organic molecule must be anionic to be retained. In the presence of inorganic anions such as chloride, perchlorate, thiosulfate, sulfate etc., at concentrations higher than 10^{-4} M, the preconcentration efficiency of carboxylates is reduced. This may be due to the competition of the inorganic anions for the copper matrix. All these results indicate that the preconcentration mechanism probably occurs through electrostatic attraction between the copper and the anion.

c) Elution of carboxylic acids

The carboxylic acids must be protonated to be compatible with the subsequent reversed phase separation column.[5] In addition, an anion must be present in excess to compete with the enriched carboxylates to allow for their elution. Thus, for eluting carboxylates, the mobile phase chosen was a mixture of aqueous acetic acid, 5% and methanol (60:40).

In this way, we compared results from a loop injection of a 20 μl solution containing a mixture of benzoic (1 μg) and anthranilic (1 μg) acids with that of the same quantity in a 10 ml sample, i.e. 500 times more dilute, which was first preconcentrated on the pre-column and

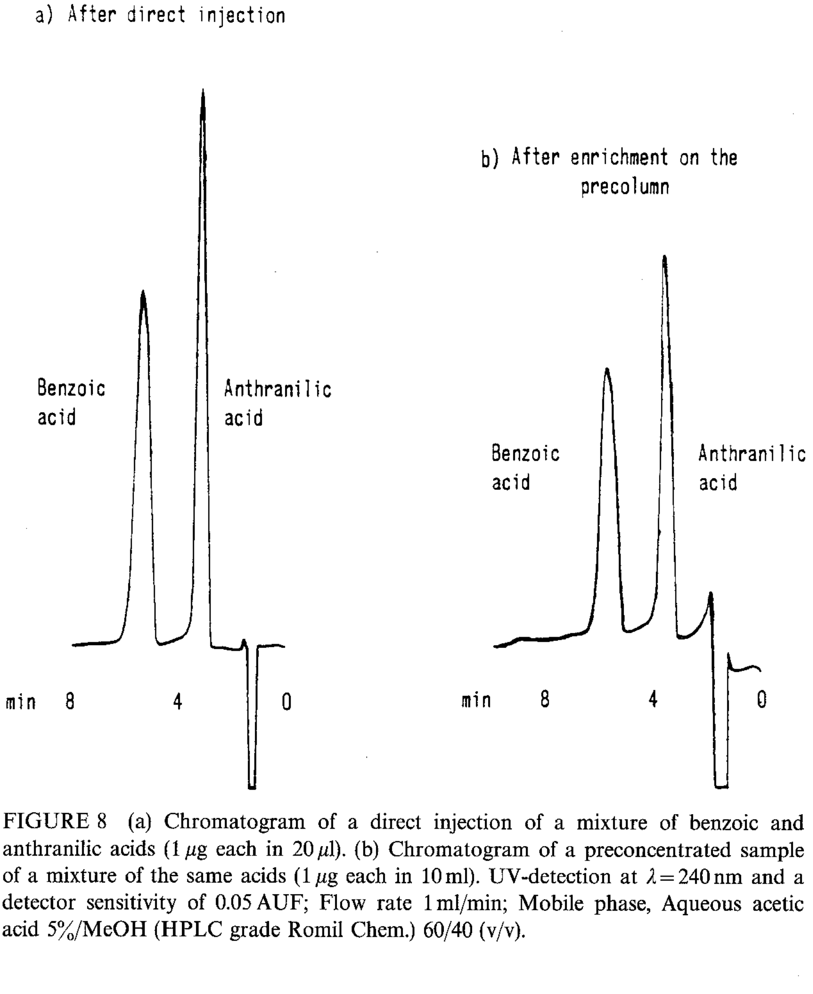


FIGURE 8 (a) Chromatogram of a direct injection of a mixture of benzoic and anthranilic acids (1 μg each in 20 μl). (b) Chromatogram of a preconcentrated sample of a mixture of the same acids (1 μg each in 10 ml). UV-detection at $\lambda = 240$ nm and a detector sensitivity of 0.05 AUF; Flow rate 1 ml/min; Mobile phase, Aqueous acetic acid 5%/MeOH (HPLC grade Romil Chem.) 60/40 (v/v).

then eluted for analysis. Results, as shown in Figure 8, reveal that well separated peaks were obtained and that recovery of the preconcentrated sample was about 75 to 100%. It should be pointed out that recovery of benzoic acid (100%) was quite better than that of anthranilic acid (75%). We are still investigating why the recovery of anthranilic acid is lower.

CONCLUSIONS

Our new copper-silica surfaces show the following characteristics

a) good stability;

b) strong binding properties with organic compounds containing heteroatoms (N, O, ...);

c) retention is not influenced by the ionic strength, but, in the case of carboxylic acids (anionic form), an excess of inorganic anions may interfere.

Finally, samples containing ng quantities of organic compounds do not show any band broadening in the chromatograms while at the same time they give good recoveries (~80%).

Further research is being carried out to synthesize other metal-loaded surfaces in view of applying them toward the pre-concentration of different components that may be found in natural systems.

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