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Hydrophobic interaction chromatographic separation of proteins in human blood fractions hyphenated to atomic spectrometry as detector of essential elements

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Abstract

The binding of metals to proteins in blood fractions was investigated applying hydrophobic interaction chromatography (HIC) for protein separation and graphite furnace atomic absorption spectrometry (GFAAS) as the element specific detector. For the semi-preparative separation of metalloproteins in erythrocytes and blood plasma, a HIC column (Fractogel EMD Phenyl I (S) 150 mm×10 mm I.D.) was adapted. The separation column was calibrated with the same four standard proteins as used in Pomazal et al. [Analyst 124 (1999) 657]. The sample injection volume and the ammonium sulphate gradient set-up were optimized: 20 or 200 μl, respectively, of blood plasma and of lysed erythrocytes were injected. The separated proteins were collected in 4-ml fractions and analyzed by GFAAS off-line. An optimization of the GFAAS measuring parameters for Cu, Mn, Fe, Zn, Co, Ni, and Cr was performed. For each element, a specific temperature program was optimized with respect to the matrix of the HIC eluate (0.02 *M* NaH_2PO_4, 1.8 *M* $(NH_4)_2SO_4$). The obtained metal profiles of the eluate were compared with the HIC chromatograms. The limits of detection (LOD) for the elements by GFAAS were: 0.5 ng Cu/ml; 0.2 ng Mn/ml; 1 ng Fe/ml; 0.2 ng Zn/ml; 0.12 ng Co/ml; 0.2 ng Ni/ml; 0.16 ng Cr/ml. The GFAAS method enabled the detection of the proteins of interest via the metals.

Keywords: Graphite furnace atomic absorption spectrometry; Blood fractions; Proteins; Metals

1. Introduction

Atomic spectrometric methods are frequently used for the specific determination of the mostly very low element concentrations in biological samples. For most of the matrices concerned, inductively coupled plasma-atomic emission spectrometry (ICP-AES) and graphite furnace atomic absorption spectrometry (GFAAS) are methods which enable a precise determination of the elements in question after a chromatographic separation of the proteins [1–8]. This paper completes the series of ICP-AES measurements described in Refs. [9–11] using the GFAAS method for the determination of metals in blood and blood fractions. In these matrices the ranges of copper, zinc and manganese concentrations are very low. For the elements of interest GFAAS allows detection limits in ppb range and was therefore selected as the method for the measurement.

Antioxidant enzymes regulating the metabolism of oxygen free radicals, superoxide dismutases (SODs), and glutathione peroxidase (GPX), are known to contain the essential trace elements Cu, Zn, Mn, and Se [12–20]. Element species of Fe and Cu or other trace metals like Co, Cr, and Ni may also affect the balance of pro-oxidants and antioxidants in blood.

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These elements are also of great importance because they are contaminants of medical alloys used for implants [21,22].

The aim of this work was to optimize the GFAAS method for each element of interest in the complex eluate matrix and to compare the results of the SEC separation and the SEC/ICP-AES hyphenation described in Ref. [9] with the results obtained by the HIC/GFAAS method [23].

2. Experimental

2.1. Chemicals

All chemicals used were reagent-grade. Ficoll-Paque® from Pharmacia Biotech; Heparin Immuno® 5.000 I.E. ml^{-1} from Immuno; nitric acid Suprapur®, hydrochloric acid Suprapur®, hydrogen peroxide® (30% H_2O_2) per Analusi (p.A.), Triton X-100, NaH_2PO_4 p.A., NaCl p.A., $Mg(NO_3)_2$ p.A., and Titrisol® single element standards from Merck, Darmstadt, Germany. The instruments used (HIC: Merck–Hitachi system, Tokyo, Japan; GFAAS: PE, Norwalk, USA) are described in Tables 1 and 2.

2.2. Sample preparation

All experiments for optimization of the method were carried out using blood samples of one female subject, prepared for analysis as described previously in Ref. [9] to avoid systematic deviations. Blood samples were collected by Venflon® and the first 3 ml were discarded to avoid metal contamination. Blood plasma and erythrocytes were separated by centrifugation (1200 *g* for 10 min). After washing the cells with a physiological sodium chloride solution they were hemolysed by a (1+9) dilution with bidistilled water and stored at −20 °C for further use.

For HIC analysis, the lysed erythrocytes were thawed, homogenized by a Vortex®, centrifuged at 1200 *g* for 10 min and filtered by a Micro Prep-Disc Filter (BIO-RAD, PTFE membrane, pore size 5 μm) prior to injection. The plasma was thawed, homogenized and centrifuged as well.

For the GFAAS off-line analysis, 4-ml fractions of the eluate after the HIC separation were collected and pipetted directly by the autosampler into the

Table 2
GFAAS apparatus

Instrument	PE 4100 ZL
Spectrometer	Littrow-design
Grating (lines mm^{-1})	1800
Atomizing unit	Transverse heated graphite tube
Background correction	Longitudinal Zeeman Effect
Magnetic field (Tesla)	0.9
Inert gas, inlet gas flow	Argon, 250 ml/min
Alternative gas, inlet gas flow	Air, 250 ml/min
Autosampler	AS-71
Sample volume (μl)	20

Table 1
HIC system

Apparatus	
Pump	Merck Hitachi L-6220
Column	Fractogel EMD Phenyl I (S) 150 mm×10 mm I.D.
Detector	Merck Hitachi UV–Vis L-4000
Fraction collection	Merck Hitachi L-5200
Separation conditions	
Injection volume	20 μl, 200 μl
Flow-rate	30 min gradient mode, 1.0 ml/min,
Eluate	0.02 *M* NaH_2PO_4, 1.8 to 0 *M* $(NH_4)_2SO_4$, *T*=30 °C, pH 6.8
Wavelength	240 nm
Fraction volume	4 ml

graphite tube. All samples and standard solutions were prepared using automatic Eppendorf pipettes.

2.3. HIC

The following parameters were optimized for the separation (pH: 6.5–7.2; temperature: 25, 30, 35 °C; injection volume: 20–500 μl; flow-rate: 0.5–2 ml/min; salt concentration: 0.1–0.5 *M* NaCl). The parameters selected are listed in Table 1. The time function for the gradient, changing the concentration of ammonium sulfate from 1.8 to 0 *M*, is shown in Fig. 2. The hand filled column was tested using myoglobin, conalbumin, ovalbumin, and bovine serum albumin (BSA). Solutions of 0.25 mg protein/ml were injected. All chromatograms were detected at a wavelength of $\lambda = 240$ nm.

Samples of blood plasma and lysed erythrocytes were injected into the column. The proteins were separated using the optimized parameters. To obtain the chromatogram of the protein separation in blood plasma 20 μl of the diluted sample ((1+3) with bidistilled water) were injected. For the determination of the metal profiles 200 μl of the water diluted sample (1+1) were injected. In both cases, the protein detection was performed by UV measurement.

2.4. GFAAS

The instrumental conditions for GFAAS were carefully selected to eliminate interferences occurring due to the high salt concentration in the eluate. Temperature curves were registered in order to select the optimal pretreatment and atomization conditions for the elements of interest.

After drying steps (1 and 2) of the temperature program, the elevated background absorption signal caused by the matrix effects of the solution resulting from the HIC protein separation was minimized by ashing the samples at 600 °C (program steps 3 and 4).

The pretreatment and the atomization temperature for the metals determined were optimized. The absorption data resulting from experiments with varying temperature for step 5 keeping the temperature of step 6 constant or varying temperature for step 6 keeping the temperature of step 5 constant, were used for choosing an optimal temperature program (Table 3) for the measurements of the elements in question. For these experiments the absorption signal was registered during step 6 (atomization).

For the optimization of the temperature program all experiments were performed using the eluate matrix solution and the pure single element standard solutions for comparison.

3. Results

3.1. HIC

The optimized conditions for the HIC-separation column were applied for the calibration of the column. The four proteins used are constituents of blood fractions. HIC was carried out in gradient mode. The proteins were retained according to their

Table 3
GFAAS: element specific parameters

	Wavelength (nm)	Calibration range (ng/ml)[a]	Detection limit (ng/ml)	Modifier	Pretreatment *T* range (°C)	Atomization *T* range (°C)
Cu	324.8	1–37.5	0.5	$Pd/Mg(NO_3)_2$	800–1600	1500–2300
Co	242.4	1–50	0.12	$Mg(NO_3)_2$	1000–1800	1800–2400
Cr	357.9	1–50	0.16	–	1000–1900	1800–2400
Fe	248.3	1–100	1.0	–	900–1800	1800–2400
Mn	279.5	1–37.5	0.2	–	900–1800	1800–2300
Ni	232.2	1–100	0.2	–	800–1400	1800–2500
Zn	213.9	1–10	0.2	–	700–1500	1500–2300

[a] Concentration range lies in the linear range of the method.

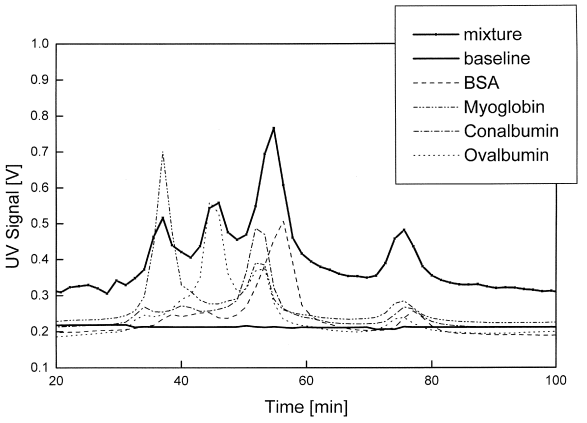

Fig. 1. Calibration of the HIC column.

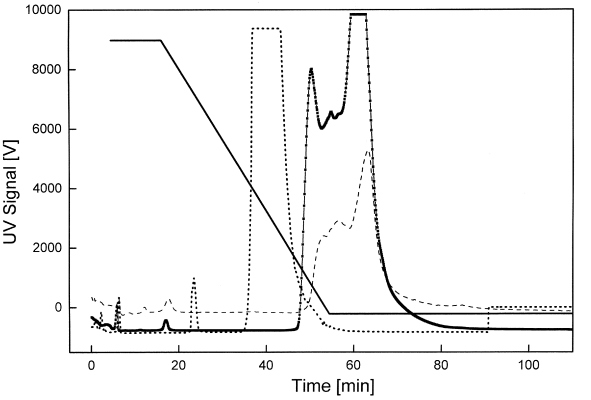

Fig. 2. Chromatograms of blood plasma and of erythrocytes: ———, lysed erythrocytes; — — —, 20 μl blood plasma; – ■ –, 200 μl blood plasma gradient mode of 1.8 to 0 *M* $(NH_4)_2SO_4$.

hydrophobic character. The chromatograms are shown in Fig. 1 (retention times: myoglobin 40 min; conalbumin 50 min; ovalbumin 57 min; BSA 63 min). The peak appearing at approximately 80 min was assumed to be a signal of a contaminant since it is occurring in the standards only but not in the samples.

In blood plasma proteins, the separation of the proteins shows the predominance of the albumins being the main constituents. Injecting 20 μl of the diluted blood plasma, the chromatogram showed protein signals, which could be identified by standard addition. This step will be carried out in the course of the next study. To visualize the proteins according to their metal content it was necessary to load the column with a high amount of the sample, in order to increase the metal concentration according to the requirements of the atomic spectrometric determinations. By the procedure applied the erythrocyte proteins were separated only partially. The main chromatographic signal belongs to hemoglobins. It was not necessary to separate the hemoglobin peak from the metal-bearing enzymes, because the metals present can be detected by the GFAAS. The identification of these proteins will be performed in the course of future investigations.

Fig. 2 shows the corresponding HIC chromatograms for 20 and 200 μl of blood plasma and for 200 μl of lysed erythrocytes. The corresponding metal profiles, which are the mean values of three different determinations, are shown in Figs. 4 and 5.

3.2. GFAAS

Using the GFAAS method for detection of metals in the eluate solution after a HIC separation of proteins makes it necessary to eliminate or minimize the effects of the matrix to the analyt signal. The high amount of the buffer and gradient salts in the eluate causes a very high background absorption signal. Ashing the samples in the graphite tube at 600 °C supported by air helps to overcome the background interactions. Furthermore for each element of interest, the pretreatment and atomization temperature were optimized.

3.2.1. GFAAS determination of Fe, Cu, Zn, and Mn

The optimization of the pretreatment and the atomizing temperature is shown using the example of Cu and Mn. Fig. 3a,b shows the resulting temperature curves for Cu and Mn in the eluate matrix and in a pure single element standard. During atomization, the matrix does not cause any remarkable change in the analyt signal. For both elements the matrix causes a lowering of the analyt signal during atomization, if the pretreatment temperature is not optimized properly. The selected pretreatment temperature for Cu and Mn was 1200 °C. The atomizing temperature was 2000 °C for Cu and 1900 °C for Mn. For Zn the matrix was pretreated at 700 °C and the atomization temperature was selected at 1800 °C.

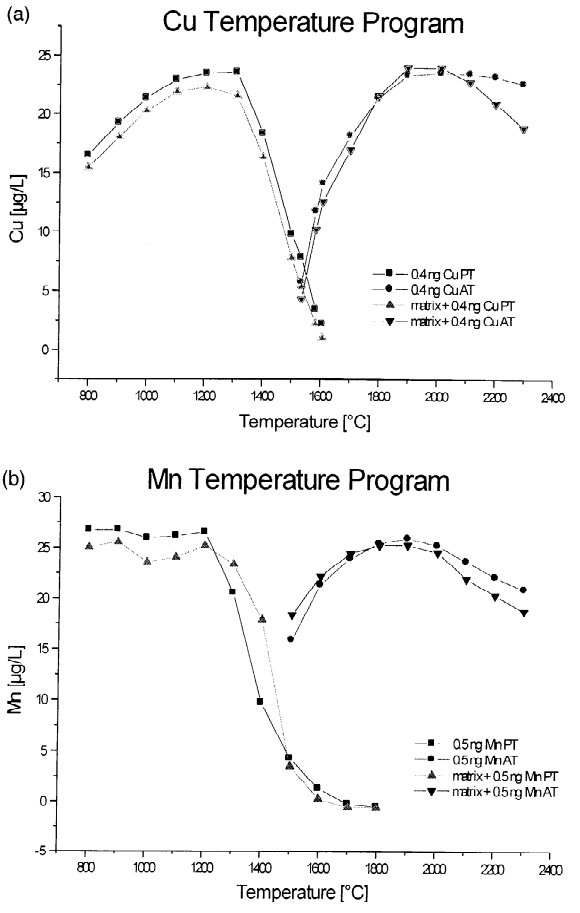

Fig. 3. (a) Optimization of Cu temperature program; (b) Optimization of Mn temperature program.

For Fe, the matrix was pretreated at 1400 °C and an atomization temperature of 2100 °C was used. In erythrocytes, Cu, Mn, and Zn were determined by GFAAS, since Cu/Zn SOD and Mn SOD are present in this blood fraction.

The collected eluate fractions after HIC separation (4 ml) were analyzed. By analyzing the amounts of Cu and Zn in the fractions and by application of a single protein standard the Cu/Zn SOD in erythrocytes could be identified. The profiles of Cu, Zn and Mn are shown in Fig. 4a.

In blood plasma fractions Cu and Mn were determined. Fig. 4b shows their profiles.

Fe was measured in both blood fractions. Fe was found in eluate fractions of erythrocytes corresponding to the hem-group signal of the HIC chromatogram. The results also enable a control of the results obtained by the optimized hyphenated method SEC/ICP-AES [9]. In Fig. 4c the profiles of Fe in erythrocytes and in blood plasma are shown.

3.2.2. GFAAS determination of Cr, Co, and Ni

The metals Cr, Co, and Ni were determined in proteins of erythrocytes as well as in blood plasma. The growing interest in analyzing these elements in blood and human fluids was the reason to investigate the possibility of identification of these metal-bearing proteins. Fig. 5a,b shows the profiles of these elements in erythrocytes and in blood plasma, respectively.

4. Discussion

The partial protein separation by HIC can be used for the detection of Cu/Zn- and Mn-bearing proteins. It is not necessary to completely separate the main peak of hemoglobin in erythrocytes or of albumin in blood plasma from other proteins, since the presence of the metal allows the detection of the proteins of interest. From the biochemical point of view the Cu/Zn-SOD could be detected directly in the separated erythrocyte proteins, without the application of complex isolation methods [9,10]. For the identification of this protein standard addition has to be applied.

GFAAS is a suitable method for the detection of metals in blood fractions. This method can be applied after the usage of separation systems for proteins based on phosphate buffers including high salt concentrations of the eluate. Proper optimization for these matrices enables the detection of very low analyt concentrations.

Ni, Cr and Co could be detected in the eluate fractions as well, but until now the corresponding proteins have not been identified.

Comparing the calibration chromatograms of the standard proteins for the two separation procedures, the separation of the proteins by the SE column is more effective than a separation by HIC. In Fig. 2 the chromatograms of blood plasma and of the lysed erythrocytes are shown. The retention times of the hemoglobin and of the albumin fraction differ sig-

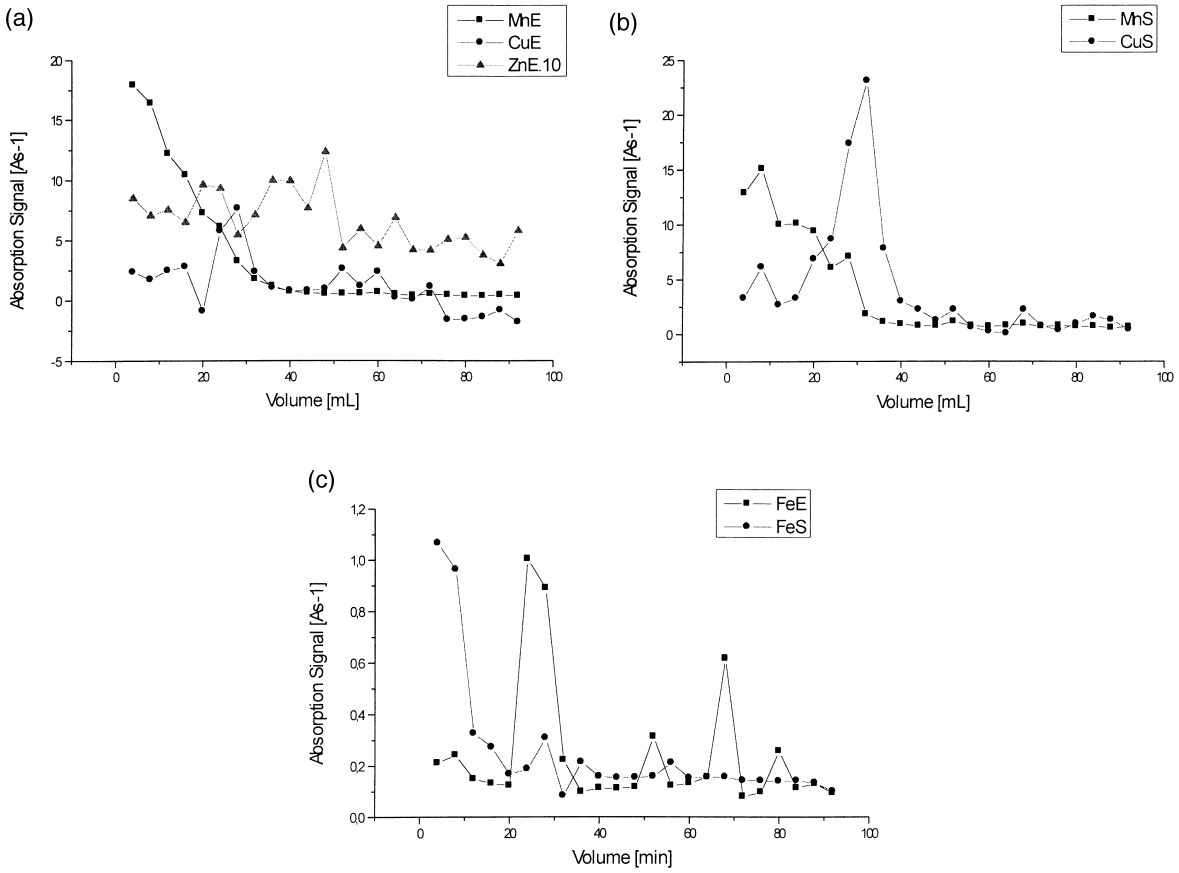

Fig. 4. (a) Cu, Zn, and Mn in erythrocytes after HIC separation; (b) Cu and Mn in blood plasma after HIC separation; (c) Fe in erythrocytes (FeE) and in blood plasma (FeS) after HIC separation.

nificantly. In Ref. [9] (Fig. 3) similar resolution could be observed using SEC for the protein separation. Applying both separation methods SEC or HIC, respectively, a complete separation of hemoglobin or albumin from the other proteins of interest is not necessary, because these proteins can be detected by the metals they are bearing. In addition to the separation principle the dimensions and volume of the two columns used are the most important distinction criteria. The 120-ml volume of the SE column causes a relatively large dilution factor for the protein fractions. The subsequent detection of the metals is therefore defined by the LOD of the detector applied. But the eluate of the SE is compatible with both determination methods, ICP-AES and GFAAS, respectively. Additionally information about the protein size is obtained using SEC. The volume of the HI column is approximately a quarter of that of the SE column and better suited for subsequent metal detection by GFAAS. The usage of the ammonium sulfate gradient requires a special sample introduction system for ICP-AES, but does not cause serious problems for the direct GFAAS determination of the metals.

SEC separation of the blood proteins is to be preferred for an on-line coupling to the ICP-AES determinations of the metal-bearing proteins present in relatively high concentration, e.g. iron in hemoglobin [9]. Since in most cases the metal concentration is very low, HIC is advantageous for the separation of the blood proteins prior to off-line GFAAS metal determination.

For the selection of the metal detector the possibility for and the advantages of an on-line or off-line

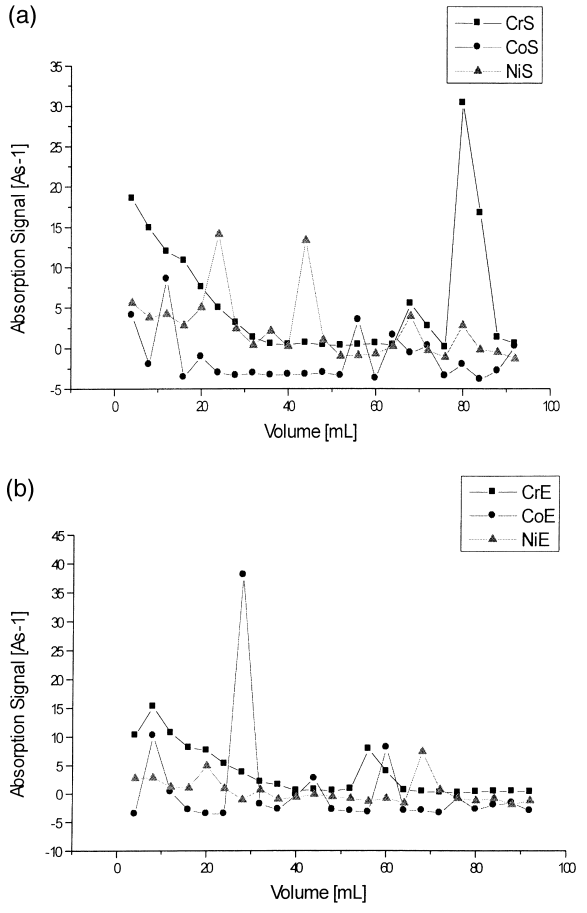

Fig. 5. (a) Cr, Co, and Ni in blood plasma after HIC separation. (b) Cr, Co, and Ni in erythrocytes after HIC separation.

coupling, respectively, must be considered [23]. In a previous paper [9], iron was detected on-line by SEC/ICP-AES in erythrocyte proteins. The hemoglobin peak was observed at the same retention time as the iron signal. On the other hand, copper and zinc were detected off-line in one fraction of the erythrocyte protein separation [9], i.e. the corresponding fraction of the chromatographic signal of the Cu/Zn-SOD, whereas in this study the Cu/Zn-SOD was detected off-line by the HIC/GFAAS hyphenation. The chromatographic dilution of the metals caused by the large volume necessary for elution using HIC is a disadvantage for both detection methods. The off-line detection of a metal by GFAAS is possible in small volumes (100 μl). The LODs of GFAAS in ppb range are lower by two orders of magnitude compared to ICP-AES, because of the possibility of direct sample injection into the graphite tube. The nebulizing technique of the sample introduction system of the ICP-AES method allows on-line coupling, but means a loss to the drain of up to 98% of the sample.

SEC and HIC can both be hyphenated to ICP-AES or GFAAS for the detection of Cu-, Mn-, Zn-, and Fe-bearing proteins. For other clinically important trace elements in blood like Ni, Cr, or Co the detection of the protein–metal complexes can reasonably be obtained using the off-line hyphenation of HIC and GFAAS only.

After intense biochemical research, the detection of proteins bearing clinically interesting metals should be possible by a proper coupling of chromatographic separation methods with atomic spectrometric element specific detection.

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