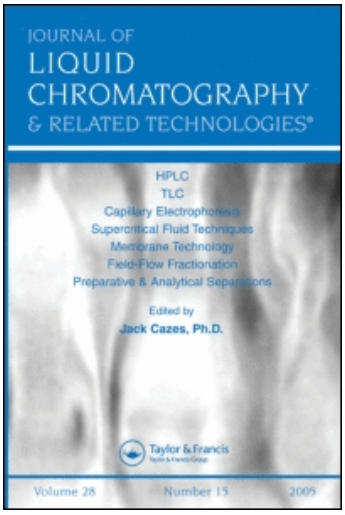

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SIMULTANEOUS DETERMINATION OF HEAVY METALS IN BOVINE LIVER AND OYSTER TISSUE BY SOLVENT EXTRACTION-REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatographic method for the simultaneous determination of ppm levels of cadmium, nickel, lead, zinc, cobalt, copper and bismuth in biological samples has been developed. Each 250 mg of Bovine Liver(NBS 1577) or Oyster Tissue(NBS 1566) was ashed in a muffle furnace over night at 500 to 550°C. Then the ash was treated with 1.5 ml of 2 N hydrochloric acid, and the solution was put into a separatory funnel. The dissolved heavy metals were extracted into chloroform as hexamethylenedithiocarbamato chelates. The metal chelates were separated on a reversed phase column(5 μm, ODS, 4.6 × 150 mm), and determined by measuring the peak height of each metal chelate. Cd, Ni, Pb, Zn and Cu were determined accurately over the concentration range of 0.5-850 ppm with standard deviation ca. 7%.

INTRODUCTION

High performance liquid chromatography(HPLC) has been used mainely for the separation of various organic materials, but in recent years, the separation and determination of inorganic materials has been reported(1).

Dithiocarbamates(2-9) and other chelating agents(10,11) have been used for the determination of heavy metals in water(5,6) and alloy(6). Edward-Inatimi(9) determined Cu, Ni, Pb and Mn in industrial effluents, standard kale and standard fish meal as diethyldithiocarbamato(DDTC) chelates. Unfortunately the separation of

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metals as chelates is not complete because the Zn chelate peak overlaps with Cu and Ni chelates. Thus the presence of Zn precludes determination of Cu and Ni.

A simple HPLC method for the simultaneous determination of heavy metals in water(12,13) and Orchard Leaves(NBS 1571) using dithiocarbamates as chelating agents has also been reported. In this paper, the authors reported a simple and accurate method for the simultaneous determination of Cd, Ni, Pb, Zn, Co, Cu and Bi in Bovine Liver and Oyster Tissue by reversed phase HPLC using hexamethyleneammonium hexamethylenedithiocarbamate(HMA-HMDC) as chelating agent.

MATERIALS

Reagents

All chemicals were analytical grade unless otherwise stated.

Standard reference materials(Bovine Liver and Oyster Tissue) were obtained from National Bereau of Standard(NBS, U.S.A.).

Two molar ammonium chloride-ammonia buffer solution(pH 7.5) was prepared as follows. 107 g(2 moles) of ammonium chloride was dissolved in ca. 900 ml of water which was then adjusted to pH 7.5 with 25% ammonia water(super special grade) and diluted with water to 1000 ml. Heavy metals were removed from this solution by extraction. Fifty ml of 0.1% diphenylthiocarbazone(dithizone)-chloroform solution was added and stirred vigorously by use of a magnetic stirrer. The aqueous phase was then transferred to another 1 l beaker and stirred with 50 ml of chloroform. This procedure was repeated until the chloroform phase did not give green color.

One molar ammonium citrate(pH 9.3) was prepared by a procedure similar that described above.

HMA-HMDC was synthesized by a modified method of Busev et al. (15). Two moles(124 ml) of hexamethyleneimine(Aldrich) and ca. 400 ml of benzene were added to a 1 l beaker placed in an ice bath. One mole (60.1 ml) of carbon disulfide was then added from a separatory funnel over 30 min under a nitrogen gas stream. Stirring was accomplished by a glass bar, and benzene was added when formation of

HMA-HMDC made stirring difficult. The white crystals formed were filtered through a glass filter, and washed 2 times on the filter with ethyl ether. Methyl alcohol was then added to the filtered crystals until ca. 80% of the crystals were dissolved. This mixture was filtered and the filtrate put into a 2 l beaker. Ethyl ether(1/3 volume of the filtrate) was added to the filtrate. Recrystallization was accomplished by standing over night in a refrigerator. The white flocculent crystals formed were filtered through a glass filter and washed 3 times on the filter with ethyl ether, then dried in a desiccator(silica gel). The dried crystals were ground to a powder to facilitate dissolution in water. The purity and structure of the crystals(HMA-HMDC) were confirmed by titration with a copper standard solution, melting point, elemental analysis, mass spectrometry and N.M.R.

The mixed metal standard solution for the analysis of Bovine Liver(A) and Oyster Tissue(B) were prepared as follows. The 500 ml of metal standard solution(A) was prepared, which contained 0.1 M hydrochloric acid(pH 1), 13.5 ng/ml(ppb) of Cd(II), 17 ppb of Pb(II), 6.5 μg/ml(ppm) of Zn(II), 9.65 ppm of Cu(II), 9.0 ppb of Co(II) and 0.8 ppb of Hg(II). The metal standard solution(B) for Oyster Tissue contained 175 ppb of Cd(II), 51.5 ppb of Ni(II), 24 ppb of Pb(II), 42.6 ppm of Zn(II), 20 ppb of Co(II) and 3.15 ppm of Cu(II). Five ml of each standard solution containes the same amount of the metals as 250 mg of Bovine Liver or Oyster Tissue samples as calculated from their certified values.

Apparatus

A liquid chromatograph consisted of a Model 6000 A pump(Waters Assoc.), a U6K universal injector(Waters), UVIDEC 100-III spectrophotometer(Japan Spectroscopic Co., Tokyo, Japan) and a Model 056 recorder(Hitachi Ltd., Tokyo, Japan) were employed. The detector was fitted with a 10 μl flow-cell volume of 10 mm pathlength and samples monitored at 260 nm.

A 4.6 × 150 mm Cosmosil 5 C_{18} packed column(5 μm, ODS, Nakarai Chemicals, Kyoto, Japan) was used. This column was immersed in a

water thermostated bath consisting of a Model 150 L cool pipe and a Model Ace 80 thermominder(Taiyo Scientific Industrial Co. Ltd., Tokyo, Japan).

A Hitachi Model 200-20 spectrophotometer was employed for the measurement of UV and visible spectra of metal-HMDC chelates and determination of their molar extinction coefficients.

A Model SA-31 auto shaker (Yamato Scientific Co. Ltd., Tokyo, Japan) was used for extraction of HMDC chelates.

METHODS

Extraction Conditions

In our previous work(13), 50 ml of aqueous sample of heavy metals were extracted into 1 ml of chloroform as the metal-HMDC chelate. When the dry ash method was employed for a biological sample, 0.1-1 g of the sample were frequently collected. Sample and chloroform volumes were modified to 25 ml and 0.5 ml, respectively in order to concentrate the metal-HMDC chelates in the chloroform phase. This modification in preveous work(13) required some investigation for optimization of the extraction conditions. Optimums of HMA-HMDC concentration and shaking time were investigated for the simultaneous quantitative extraction of Cd(II), Ni(II), Pb(II), Zn(II), Co(II), Cu(II), Hg(II) and Bi(III). Peak height were measured at various volume of HMA-HMDC solution and shaking time with metal concentrations in the range of 10-1000 ppb.

Stability of Some Dithiocarbamates

Both dithiocarbamates and some of their chelates are unstable in reversed phase mobile phases such as acetonitrile/water and methyl alcohol/water under ambient conditions. However, it was found that addition of the chelating agent to mobile phases suppressed the decomposition of the some dithiocarbamato chelates such as those of cadmium, lead and zinc. Because the chelating agent added is also unstable in the mobile phase, decomposition of Na-DDTC, ammonium pyrrolidinedithiocarbamate(APDC) was investigated according to the procedure in Figure 1. The time course of the decomposition of the three dithiocarbamates was also examined.

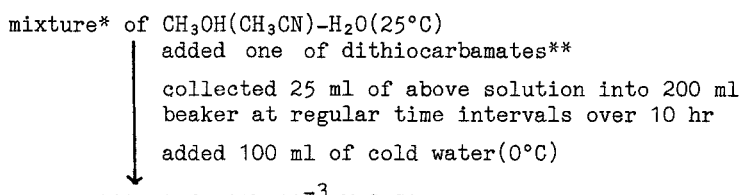

FIGURE 1 Procedure for the Conductmetric Titration of Dithiocarbamates(Na-DDTC, APDC and HMA-HMDC)

* : methyl alcohol(acetonitrile)/water=70/30
** : Concentration of each dithiocarbamate was 8×10^{-3} M in the mixture

UV and Visible Spectra of Metal-HMDC Chelates

UV and visible spectra of eight metal-HMDC chelates were measured by following the procedure shown in Figure 2.

Molar extinction coefficient at the maximum absorption wavelength was also measured. The reference(blank) solution was fleshly prepared because its absorbance increased slowly with time.

HPLC Separation of Eight Metal-HMDC Chelates

In our earlier work(12), Ni(II), Pb(II), Co(II), Cu(II), Hg(II) and Bi(III) have been separated as APDC chelates. Cd(II), Ni(II), Co(II), Cu(II), Hg(II) and Bi(III) have been also separated as HMDC chelates(13), but Pb(II) and Zn(II) chelates could not be separated from Ni(II) chelate.

In order to separate eight metal-HMDC chelates described above, an ODS column(Cosmosil 5 C_{18}, 4.6 × 150 mm) was employed. Various solvents such as diethyl ether, ethyl acetate, ethyl alcohol, acetonitrile, water and buffer solutions were examined as mobile phases.

Addition of 1.0×10^{-3} M HMA-HMDC to the mobile phase was needed to obtaine reproducible chromatograms of HMDC chelates. Peak heights of the HMDC chelates were measured at various HMA-HMDC concentration in order to determine the optimum concentration of HMA-HMDC in the mobile phase.

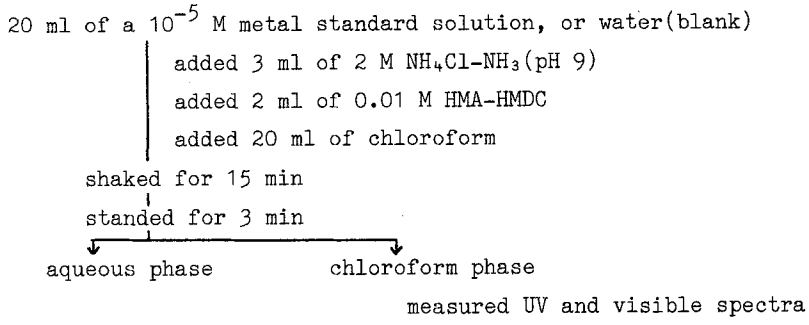

FIGURE 2 Extraction Procedure for Measuring UV and Visible Spectra of the Metal-HMDC chelates

Effect of column temperature on the separation of HMDC chelates was also investigated over the range of 15-50°C.

Working Curves

It was found that the working curves for studied heavy metals HMDC chelates have good linearity over the concentration range of 0.3-2000 ppb(13). When heavy metals in 250 mg of a biological sample were dissolved in 25 ml of aqueous phase, this concentration range corresponds in the range of 0.03-200 ppm in a original sample.

The working curves for Bovine Liver and Oyster Tissue were made as follows; 5 ml of mixed metal standard solution(A) or (B)(see Reagents) and 20 ml or 25 ml of water(blank) were first put into a separatory funnel. After extraction, 5 µl of the chloroform extract was injected into the HPLC column. Each working curve was made with only the two plots(standard and blank).

Ashing Method for Bovine Liver and Oyster Tissue

The dry ash method was employed for the two biological samples because it is simple and has no reagent blank except a mineral acid required for dissolution of the metals in the ash. Ashing temperature was investigated over the range of 350-600°C. Dry ashing was carried out over night for convenience.

RESULTS AND DISCUSSION

Extraction Procedure

Effect of the amount of HMA-HMDC on extraction of Cd(II), Ni(II), Pb(II), Zn(II), Co(II), Cu(II), Hg(II) and Bi(III) is summarized in Figure 3. It was found that 1.5-4.0 ml of 0.01 M HMA-HMDC gave maximum and constant peak heights of the eight metal chelates in the concentration range of 10-1000 ppb. The effect of shaking time is given in Figure 4. Quantitative extraction of the eight metals in the concentration range of 10-1000 ppb was accomplished by shaking for 10-20 min.

Based on the above results, the following procedure is recommended(Fig. 5) for the simultaneous extraction of Cd(II), Ni(II), Pb(II), Zn(II), Co(II), Cu(II), Hg(II) and Bi(III).

Evaluation of HMA-HMDC as Chelating Agent for HPLC Analysis

The measured UV and visible spectra are shown in Figure 6. The molar extinction coefficients of the chelates are given in table 1 with DDTC and APDC chelates measured by similar methods as shown in Figure 1. Based on the above results, 260 nm was selected for the detection of HMDC chelates. Molar extinction coefficients of the HMDC chelates were larger than those of APDC and DDTC chelates, and apparent decomposition rate of HMA-HMDC in water or in mobile phase (CH_3OH-H_2O or CH_3CN-H_2O) is the smallest in the three dithiocarbamates(Fig. 7).

In view of above fact it is reasonable to conclude that HMA-HMDC is more sensitive and stable chelating agent than APDC or DDTC.

HPLC Separation of Eight Metal HMDC Chelates

Reproducible chromatograms were obtained when 10% or more 10^{-3} M HMA-HMDC and 2.4% or more 2 M NH_4Cl-NH_3 buffer solution were added to the mobile phase. Optimum column temperature for separation of the eight HMDC chelates was 30-50°C. Higher temperatures were not examined.

Best separations were obtained when a mixture of ethyl alcohol/methyl alcohol/water/10^{-3} M HMA-HMDC/2 M NH_4Cl-NH_3(pH 7.5) (=40/32/

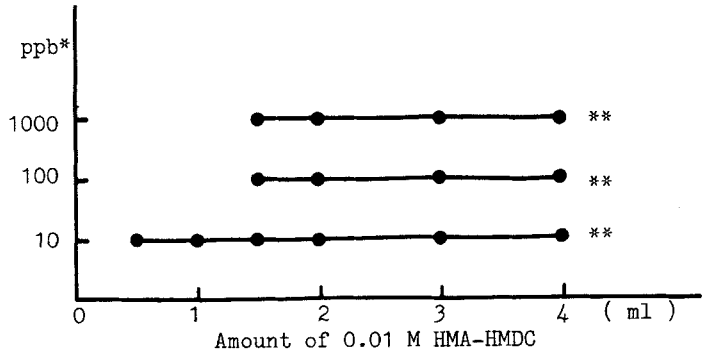

FIGURE 3 Effect of HMA-HMDC Amount on the Extraction of the Eight Heavy Metals

* : concentration of each heavy metals in 25 ml of aqueous phase
** : These lines indicate maximum and constant peak heights range for the all eight heavy metals.
shaking time : 15 min

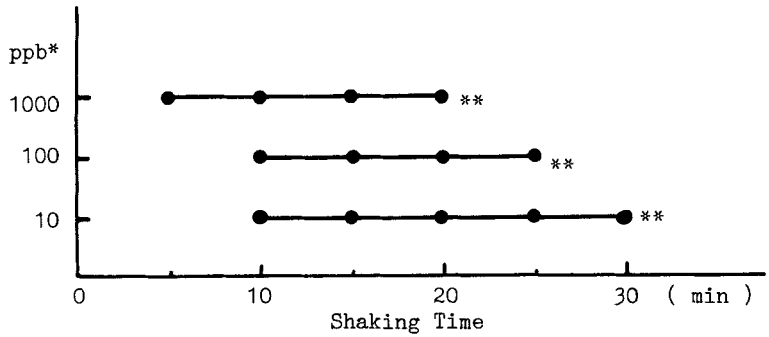

FIGURE 4 Effect of Shaking Time on the Extraction of Eight Heavy Metals

Amount of 0.01 M HMA-HMDC was 2 ml.
* : concentration of each metal in 25 ml of aqueous solution
** : These lines indicate maximum and constant peak heights range for the all eight heavy metals.

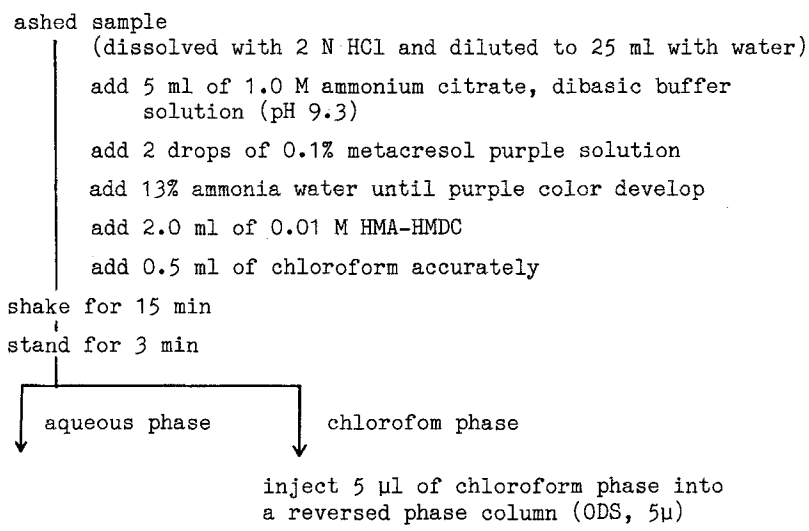

FIGURE 5 Recommended Extraction Procedure for the Determination of Cd, Ni, Pb, Zn, Cu, Co, Hg and Bi in Biological Samples

13/12/3) was used as the mobile phase. A typical chromatogram is shown in Figure 8. Although Mn(II), As(III) and Sn(II) were also extracted by the recommended procedure(Fig. 5), they did not appeared on the chromatogram. This is probably due to their low stability constants. Fe(III) was not extracted by the extraction procedure because the added citrate masked Fe(III) ion.

Determination of Heavy Metals in Bovine Liver and Oyster Tissue

When the dry ash method was employed, 1 g or more of sample was difficult to ash completely. Therefore, 250 mg of samples were used. High temperature(600°C) resulted in low analytical results for Zn and Pb. At low temperatures(350°C and 400°C) ashing was not accomplished completely. The optimum temperature for Bovine Liver and Oyster Tissue samples was 550°C and 500°C, respectively.

Porcelain crucibles and quartz boats were used for the ashing of the Oyster Tissue and Bovine Liver, respectively. It was felt

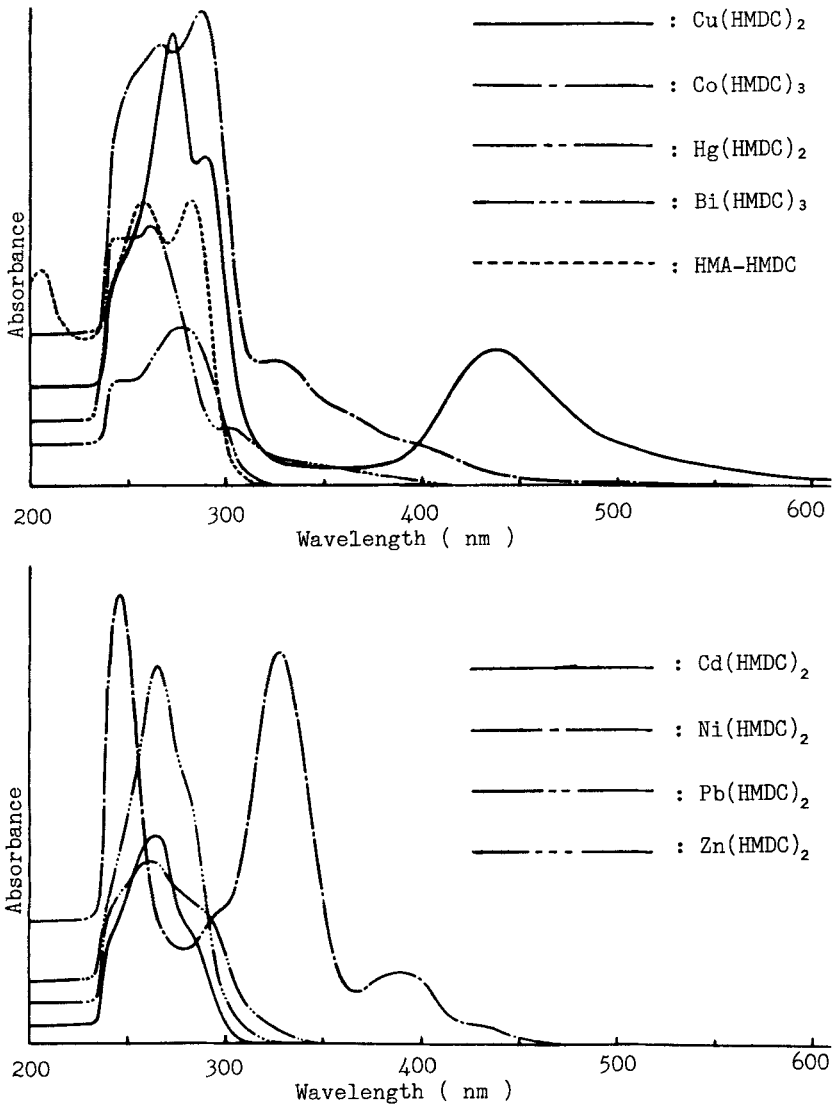

FIGURE 6 UV and Visible Spectra of Metal-HMDC Chelates

The mole ratio of above chelates was determined by the mole ratio method.

TABLE 1 Molar Extinction Coefficients of Metal Dithiocarbamates

Metal	Molar Extiction Coefficient Na-DDTC(λ max)	APDC(λ max)	HMA-HMDC(λ max)
Cd	37000 (264 nm)	35000 (261 nm)	50000 (266 nm)
Ni	37000 (247 nm)	38000 (246 nm)	43000 (246 nm)
Pb	37000 (263 nm)	43000 (260 nm)	65000 (262 nm)
Zn	33000 (264 nm)	36000 (260 nm)	36000 (264 nm)
Co	36000 (272 nm)	38000 (269 nm)	84000 (266 nm)
Cu	34000 (272 nm)	33000 (269 nm)	52000 (273 nm)
Hg	39000 (277 nm)	40000 (272 nm)	73000 (278 nm)
Bi	77000 (261 nm)	84000 (260 nm)	108000 (262 nm)

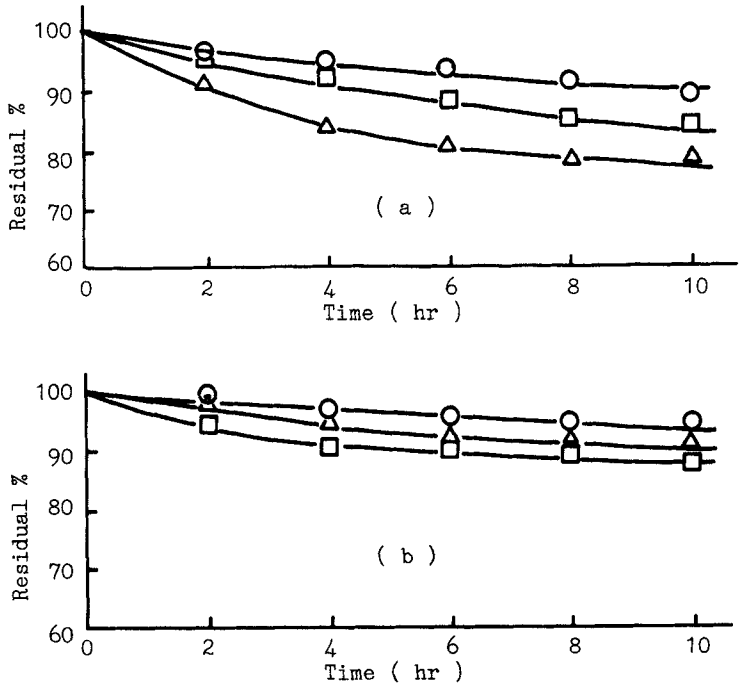

FIGURE 7 Time Courses for Na-DDTC, APDC and HMA-HMDC in Eluent

○ : HMA-HMDC, △ : APDC, □ : Na-DDTC

Experimental Conditions

(a) : CH_3CN/H_2O = 70/30

(b) : CH_3OH/H_2O = 70/30

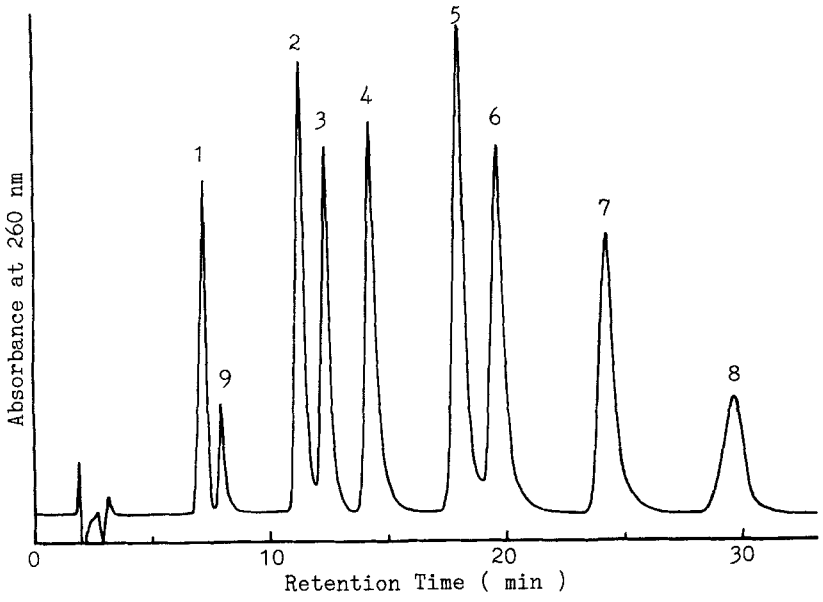

FIGURE 8 Typical Chromatogram of Metal-HMDC Chelates

1 : Cd(62.5 ng), 2 : Ni(125 ng), 3 : Pb(125 ng)
4 : Zn(62.5 ng), 5 : Cu(125 ng), 6 : Co(62.5 ng)
7 : Hg(250 ng), 8 : Bi(125 ng),
9 : decomposotion product of HMA-HMDC

HPLC Conditions
column : Cosmosil 5 C_{18}, 4.6×150 mm, 35.0±0.1°C
mobile phase : ethyl alcohol/methyl alcohol/water/ 10^{-3} M HMA-HMDC/2 M NH_4Cl-NH_3(pH 7.5) =40/32/13/12/3
flow rate : 1.0 ml/min, sample size : 5 µl
The mobile phase reservoir was cooled in an ice bath

that quartz was not necessary at 550°C or below. A boat is, however, convenient to spread a sample thinly.

The analytical results are shown in Table 2 and Figure 9. In the case of Oyster Tissue, Zn content is larger than that of Pb by a factor of about 2000. However, the separation of the chelates was good and analytical results showed good agreement with the certified values. Cobalt and bismuth were not detected because the contents are too low. Mercury probably evaporated at the ashing step.

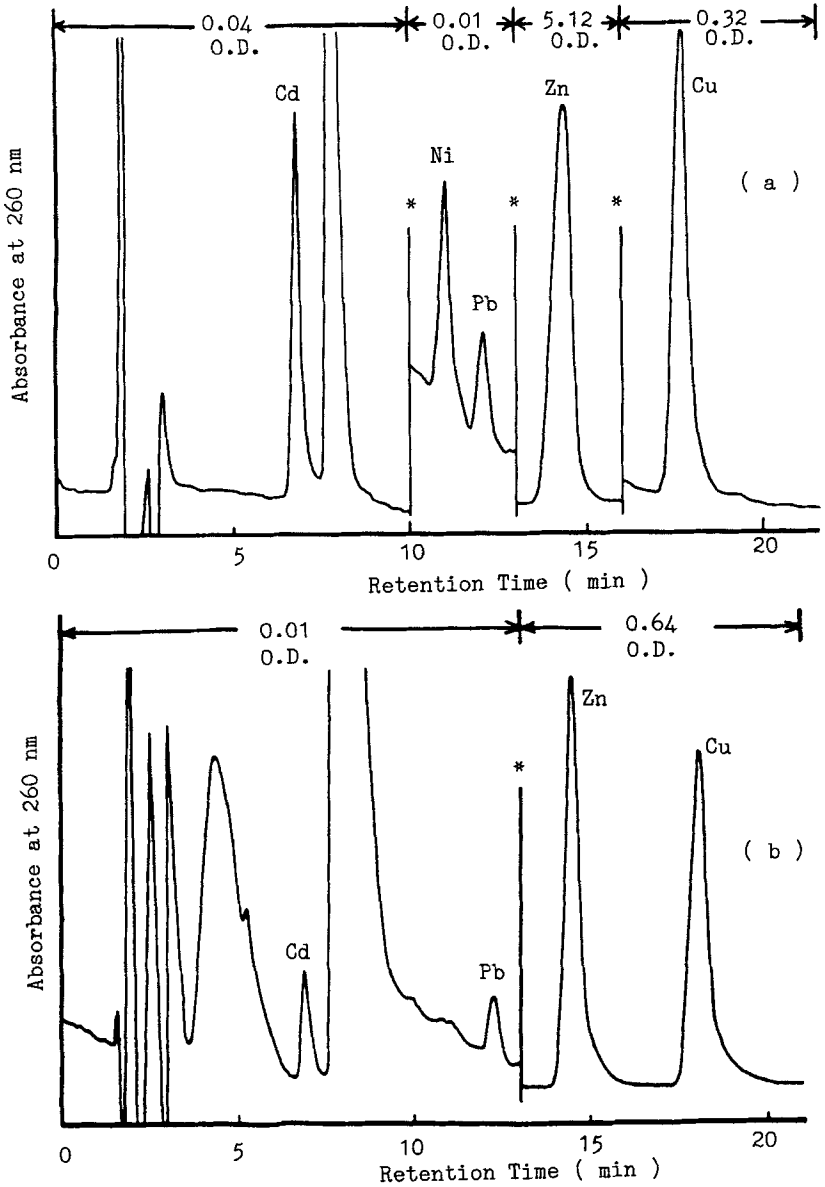

FIGURE 9 Analytical Chromatograms of Oyster Tissue(a) and Bovine Liver

* : change point of detector response
HPLC conditions were same as Fig. 8.

TABLE 2 Analytical Results of Heavy Metals in Biological Samples by Presented HPLC Method

Sample	Metal	Certified Value(ppm)	Found* (ppm)	Detector Range(O.D.)
Oyster** Tissue (NBS 1566)	Cd(II)	3.5 ± 0.4	3.2 ± 0.2	0.04
	Ni(II)	1.03 ± 0.19	0.80 ± 0.1	0.01
	Pb(II)	0.48 ± 0.04	0.44 ± 0.06	0.01
	Zn(II)	852 ± 14	848 ± 17	5.12
	Cu(II)	63.0 ± 3.5	64.0 ± 4.6	0.32
Bovine*** Liver (NBS 1577)	Cd(II)	0.27 ± 0.04	0.29 ± 0.02	0.01
	Pb(II)	0.34 ± 0.08	0.29 ± 0.03	0.01
	Zn(II)	130 ± 13	133 ± 6	0.64
	Cu(II)	193 ± 10	191 ± 7	0.64

* : average value ± standard deviation
** : N=4, ash ; 500°C-13 hr (porcelain crucible)
*** : N=8, ash ; 550°C-13 hr (quartz boat)

CONCLUSION

Heavy metals in Bovine Liver and Oyster Tissue were determined easily and accurately. Determination of heavy metals by HPLC is very simple in comparison with AAS. When AAS is employed for the determination of several metals, a lamp change is required and the working curves must be made for individual metals in the optimum concentration range. The sensitivities of flameless AAS is exellent, but it requires significant skill for reproducible results and the apparatus is more expensive than in HPLC. HPLC requires no specific skill and enables simultaneous determination of heavy metals in environmental samples as reported in this paper and previous work(13). Determination of ppm levels of heavy metals could be accomplished with preconcentration(solvent extraction).

Simultaneous determination of ppm levels of the heavy metals in plant samples is in progress.

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