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Determination of hydroxy acids as their copper(II) complexes by reversed-phase liquid chromatography with UV detection

Da Shi Lu, Wen Yu Feng, Da Hui Ling and Wang Zhog Hua

Department of Chemistry, Wuhan University, Wuhan 430072 (China)

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ABSTRACT

A method for the liquid chromatographic separation and UV detection of hydroxy acids without any pretreatment of derivatization is described An aqueous solution containing copper ion, alkylsulphonate and acetate buffer is used as the mobile phase in conjunction with a conventional reversed-phase column Detection is carried out at 254 nm The influence of pH and the concentrations of copper ion, alkylsulphonate and buffer on the retention and detection response has been investigated These parameters were found to provide powerful means of selectivity and response manipulations

INTRODUCTION

The separation and determination of hydroxy acids, such as lactic, citric, malic, tartaric and α-hydroxybutyric acids, are important problems in physiology, biochemistry, food, beverages, health and related areas For example, the concentration of lactic acid in the plasma of patients with leukaemia is much higher than that of healthy persons [1] These compounds are present as major acid components in fruit, vegetable, milk and wine [2]

Ion-exchange chromatography is suitable for analyses for hydroxy acids [3] There are several difficulties with the direct separation and determination of hydroxy acids by gas or reversed-phase liquid chromatography with UV detection because of their low volatility, strong polarity, ionicity and lack of appreciable UV absorption Derivatizations for improved separation and detectability have been utilized Derivatization with phenacyl bromide has been applied to the determination of hydroxy acids in wines using reversed-phase chromatography with UV detection [4] Rigas and Pietrzyk [5] described an indirect spectrometric chromatographic method for the determination of lactic and α-hydroxybutyric acids, etc , using iron(II)–phenanthroline as a detection reagent In our laboratory, the reversed-phase chromatographic separation and indirect UV spectrophotometric detection of hydroxy acids were studied using various detection reagents [6]

Levin and Grushka [7] reported a new approach to the determination of amino acids on a reversed-phase column using an aqueous mobile phase containing copper ion In this approach, the detection of amino acids was accomplished by the *in situ* formation of their complexes The determination of citric acid in milk using UV spectrophotometry with the formation of a complex with copper ion has been reported [8] None of studies has investigated in detail the use of copper ion in high-performance liquid chromatography for the separation and determination of hydroxy acids

This paper describes a method that allows the determination of hydroxy acids as their Cu(II) com-

Correspondence to Dr D S Lu, Department of Chemistry, Wuhan University, Wuhan 430072, China

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plexes on a conventional reversed-phase column in conjunction with a UV detector In order to ascertain the possibility of applying UV detection, the UV absorption spectra of hydroxy acid–Cu(II) mixtures in aqueous solution were determined A reversed-phase chromatographic system for the separation and determination of hydroxy acids with an aqueous mobile phase containing copper ion and alkylsulphonate was investigated An hydrophobic alkylsulphonate was added to the mobile phase as an ion-pair reagent and spectrophotometric sensitizing agent in order to increase the retention and detection response Acetate buffer was used to maintain a suitable pH and ionic strength The effects of various factors on retention and detection response were studied, especially pH and the concentrations of copper ion, alkylsulphonate and buffer Guidelines are given for optimization of the separation and detection The calibration graphs of peak area *versus* sample amount, detection sensitivity and minimum detectable amounts were also studied

EXPERIMENTAL

Apparatus

A Model UV-240 spectrophotometer (Shimadzu) was used The chromatographic system consisted of a Model LC-6A pump (Shimadzu), a Model 7125 injector with a 20-μl loop (Rheodyne), a Model DZ-1 fixed-wavelength (254 nm) UV detector (Shanghai Scientific Instrument Factory), a Type 3056 recorder and a Model C-R2A digital integrator (Shimadzu) The column used was Zorbax-ODS (250 × 4 mm I D) (DuPont) A precolumn made in our laboratory, packed with 5-μm YWG CH (Tianjing Second Chemical Reagent Factory), was connected between the pump and the injector The pH measurements were performed with a pH-3E digital pH meter (Jiansu Electroanalysis Instrument Plant) A Model CQ-50 ultrasonic cleaner (Shanghai Ultrasonic Instrument Plant) was used

Reagents and solvents

Analytical-reagent grade or equivalent chemicals and water doubly distilled in a quartz still were used Acetate buffer was prepared with acetic acid and sodium hydroxide Copper(II) acetate was obtained from Shanghai Chemical Reagent Factory and sodium hexanesulphonate from Beijing Chemical Reagent Institute

Samples

The following hydroxy acids (HA) were chosen as sample solutes lactic (LA), citric (CA), tartaric (TA), malic (MA) and α-hydroxyisobutyric acid (HBA) Stock standard solutions of the samples (0 1–0 01 *M*) were prepared and diluted as needed

Procedures

The solutions of the HA–Cu(II) complexes for determination of UV absorption spectra were made with appropriate volumes of stock of samples, copper(II) acetate and buffer, then diluted to 25 ml with water The UV absorption spectra of the solutions were determined in the range 330–190 nm The absorbances at 254 (A_{254}) and 230 nm (A_{230}) were recorded

The mobile phases were prepared by dissolving known amounts of hexanesulphonate and copper (II) acetate in the acetate buffer, adjusted to the desired pH, then filtered and degassed in an ultrasonic bath before use The flow-rate of the mobile phase was set at 1 ml/min The eluent was passed through the column for about 1 h until equilibrium was observed by recording the breakthrough curve due to the background absorption by copper ions To protect the instrument, prolong the lifetime of the column and obtained reproducible results for the retention and detection sensitivity, at the end of each working day the chromatographic system was flushed with 0 01 *M* EDTA in the buffer solution (pH 5 6), water and methanol in that order

RESULTS AND DISCUSSION

UV absorption spectra of hydroxy acid–Cu(II) complexes in acetate buffer solution

In the reversed-phase chromatographic system with a UV detector using buffer solution without copper ion as the mobile phase, no detection response of the HAs was observed Copper ion is known to form complexes with HAs, which have similar stability constants to amino acid–copper ion complexes [9] Fig 1 shows the UV absorption spectra of the HA–copper ion mixture in acetate buffer solution For comparison the absorption curves of LA, MA and copper(II) acetate are also given No

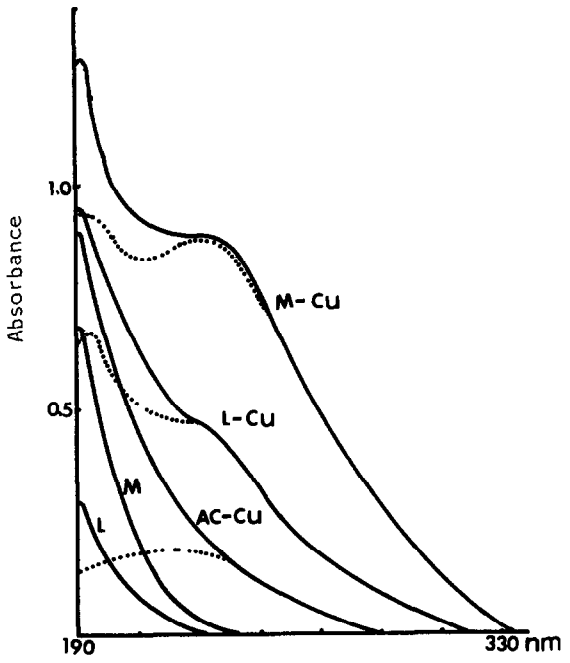

Fig 1 UV absorption spectra of HA–Cu(II) mixture in solution of acetate buffer (5 m*M*, pH 5 6) L, M and AC refer to LA, MA and acetate, respectively, Cu refers to copper ion Water was used as a reference for solid lines and acetate buffer (5 m*M*) for dotted lines

appreciable absorption is observed above 210 nm for the HAs In the presence of copper ion the absorption of the HA solutions is much stronger in the range 190–330 nm because of the formation of HA–Cu(II) complexes For the spectra of the complexes, shoulder peaks between 230 and 240 nm are observed and the absorbances at 254 nm are also very high

Table I lists the absorbances at 230 and 254 nm for the mixture solutions The absorbances of the complexes of the HAs having multiple hydroxy or carboxy groups, such as MA, CA and TA, are higher than those of the monobasic HAs, possibly because the former have a stronger complexing capacity than the latter for copper ion

Factors that affect the retention and detection response

The HAs with strong polarity and weak hydrophobicity show hardly retention on conventional reversed-phase columns and the separation selectivity based on solvophobic interaction is very poor It is believed that the formation of HA–Cu(II) complexes will occur in the chromatographic system According to evaluation of the dilution factors arising from the column, which can be expressed as a function of the column efficiency, the injection volumes and the retention volumes of the solutes [10], it is found that the concentrations of HAs in the mobile phase are slightly lower than that of copper ion, assuming that all the complexes have 1 1 composition and are positively charged, making it possible to form ion pairs of ternary complexes with hexanesulphonate Probably the reactions can be written as

$$HA + Cu^{2+} \rightleftharpoons [ACu]^{+} + H^{+} \quad (1)$$

$$[ACu]^{+} + R^{-} \rightleftharpoons ACuR \quad (2)$$

where R^{-} represents the anion of hexanesulphonate Interaction of species should enhance the retention via a dynamic ion-exchange, ion-pair partition or ion-interaction mechanism In order to compare the sensitivities under different conditions the response value, R, of detection is represented by the area of the peak produced per unit concentration of the solute injected, in units of $m^2/mmol$

Influence of Cu(II) concentration

The retention and detection response of the HAs as a function of copper ion content in the mobile

TABLE I

ABSORBANCES OF HA–Cu(II) MIXTURE IN BUFFER SOLUTION

In each case the solution contained 5 m*M* acetate buffer, 0,4 m*M* HA and 0 4 m*M* copper ion (pH 5 6)

Absorbance	LA	LA–Cu	HBA	HBA–Cu	MA	MA–Cu	CA	CA–Cu	TA	TA–Cu
A_{320}	0	0 357	0 005	0 408	0 011	1 004	0 008	1 120	0 008	0 894
A_{254}	0	0 162	0 004	0 200	0 002	0 760	0 005	0 936	0 006	0 600

phase are illustrated in Fig 2 It can be seen that the capacity factors, k', for TA and MA show a maximum values with increase in copper ion concentration, whereas there is a gradual increase for LA and HBA a smaller increase for CA, with three carboxy groups, whose hydrophobicity is less changed by complex formation When the Cu(II) concentration reaches a certain level (about 0 5 mM), no further marked increase or even a decrease in k' is observed, which can been explained by the lack of a further increase in the concentration of the complexes, and by the increase in the eluting power of the mobile phases as further increase in the concentration of copper ion, which is a participant in the dynamic ion-exchange process

Fig 2 demonstrates that the concentration of Cu (II) has a much greater effect on the detection response of polybasic acids than on that of monobasic HAs, owing to the great differences between the absorption values of the two kinds of complexes In the range of Cu(II) concentration from 0 05 to 1 mM the detection response for polybasic HAs first increases sharply to a maximum and then tends to decrease, whereas for monobasic HAs it first increases slowly between 0 05 and 0 5 mM and then decreases slightly with further increase in the concentration of copper ion

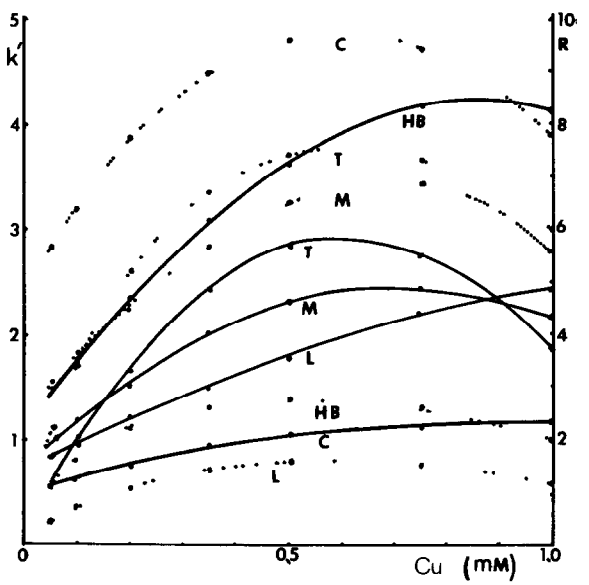

Fig 2 Effect of copper ion concentration on the retention and detection response of HAs The mobile phase contained 2 mM acetate buffer (pH 5 6) and 5 mM hexanesulphonate, temperature, 30°C, flow-rate, 1 0 ml/min, UV detection at 254 nm L, HB, C, M and T refer to LA, HBA, CA, MA and TA, respectively Solid lines, variation of retention, dotted lines, variation of detection response

Influence of hexanesulphonate concentration

Fig 3 illustrates the relationship between the retention, response of the solutes and hexanesulphonate concentration It is obvious that the addition of hexanesulphonate has a considerable influence on the retention and detection response for the HAs, consequently modifying their separation selectivity When hexanesulphonate is absent from the mobile phase the retention is lower and tends to be similar for all the HAs, owing to their weak hydrophobicity The k' values first increase rapidly for all the HAs, and then remain relatively constant for LA and HBA and tend to decrease for CA, MA and TA with increase in hexanesulphonate concentration from 0 to 10 mM The decrease in the retention of polybasic hydroxy acids with further increase in the concentration of hexanesulphonate may be due to electrostatic repulsion between the anion of hexanesulphonate adsorbed on the stationary phasae and negatively charged solutes with multiple carboxy groups The results show that the

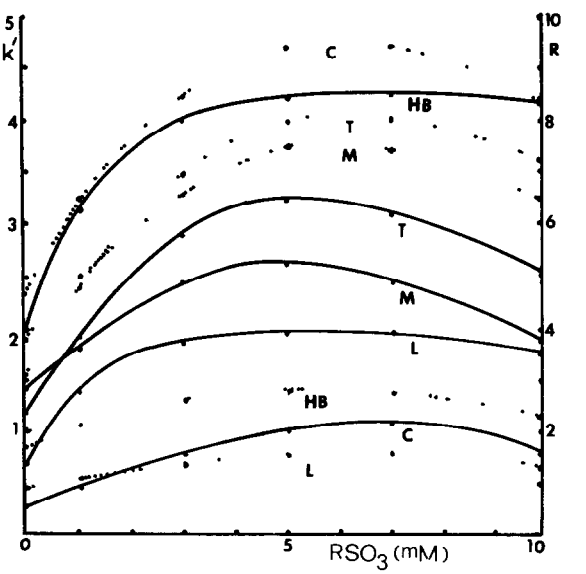

Fig 3 Effect of hexanesulphonate concentration on the retention and detection response of HAs Chromatographic conditions as in Fig 2 except 0 5 mM Cu(II) Lines as in Fig 2

retention of HAs at lower concentrations of hexanesulphonate can be regulated by the controlled addition of the latter

Fig 3 shows that the R values of polybasic HAs increase sharply as the concentration of hexanesulphonate increases from 0 to 5 mM, and then decrease with further increase in from 5 to 10 mM No significant effect of hexanesulphonate on the R values of monobasic HAs is observed, as could be expected owing to their lower capacity to form ternary complexes and their smaller absorbances

Influence of buffer concentration

The concentration of acetate buffer has an important effect on the retention and detection response under conditions of constant pH and copper ion concentration, as shown in Fig 4 The retention of polybasic HAs increases initially as the acetate buffer concentration increases from 1 to 4 mM, and then decreases slowly with further increase in concentration For monobasic HAs the retention always decreases with increase in acetate concentration from 1 to 50 mM The increase in the retention may be explained by the simultaneous formation of mixed complexes with acetate radical ion as a secondary ligand or solvation of HA–Cu(II) complexes by acetate buffer, therefore leading to the increase in the hydrophobicity of the solutes Presumably, the decrease in k' is produced by an increase in the solvent strength, which is a function of acetate content in the mobile phase, and by a decrease in the tendency of the solutes to interact by ion exchange with residual Si–OH groups on the surface of the packing [11] Experiments have shown that phosphate buffer is unusable when copper ion is present in the mobile phase The check valves of the reciprocating pump often became blocked by copper ion when phosphate buffer was used because of precipitation of copper ion by the anion of the phosphate buffer

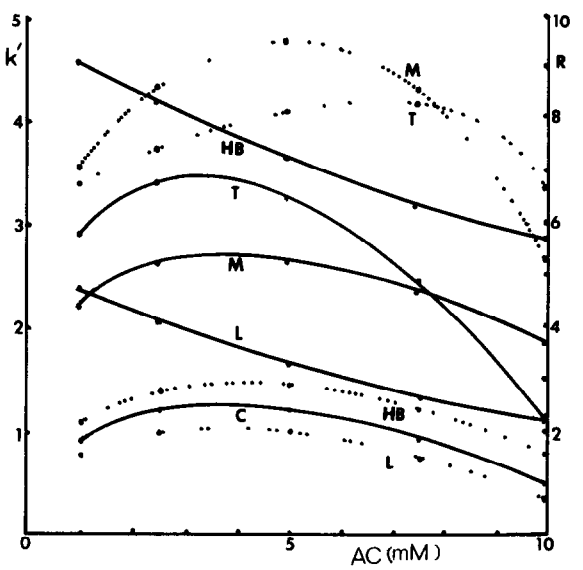

Fig 4 Effect of acetate buffer concentration on retention and detection response of HAs Chromatographic conditions as in Fig 2 except 0 5 mM Cu(II) and buffer concentration at pH 5 6 Lines as in Fig 2

Comparing the variations in R values in Figs 3 and 4, it can be seen that acetate has a similar effect to hexanesulphonate on the detection response The increase and decrease in response can probably be explained by the solvation of the complexes and the background absorptyion of copper(II) acetate Fig 4 shows that the optimum buffer concentration for the detection of the HAs is between 2 and 8 mM It is unnecessary to use concentrations above 10 mM with regard to either the separation selectivity or the detection response An important factor is that when the acetate concentration is above 10 mM, the system peak produced by acetate is so large that it will interfere seriously in the determination of the sample peaks It was found that the system peak due to acetate almost disappears if the acetate concentration is below 5 mM In addition, the column lifetime will decrease as the buffer concentration increases

Influence of pH

It is possible to change the complexation equilibria by varying the pH and therefore to influence the hydrophobicity and UV absorbance of the solutes The changes in k' and R values of the HAs with variation of pH in the mobile phase containing copper ion and hexanesulphonate are demonstrated in Fig 5 The retention of polybasic HAs increases slowly in the pH range 3 5–5 and increase sharply at pH $>$ 5 It is obvious that the formation of the complexes as the dissociation of the HAs increases enhances the hydrophobicity of the solutes The decrease in k' values of monobasic HAs with increase in pH may be caused by a lower hydrophobicity of

the complexes than that of the molecular HAs at lower pH, and by increasing the eluting power of the mobile phase with increase in concentration of OH^- ions

Fig 5 shows that the response values of all the hydroxy acids are relatively low at pH < 5, owing to a lower capability of copper ion complexing with the molecular HAs and a shift of eqn 1 to the right with increasing pH, leading to an increase in the response The drastic increase in the response at pH > 5 results from the formation of complexes which have high UV absorbances The background absorption produced by copper ion in the presence of acetate, hexanesulphonate and an excess of OH^- ion with further increase in pH causes the *R* values to decrease

On the basis of the above investigation, the HAs were chromatographed with UV detection at 254 nm using an aqueous mobile phase containing Cu (II), hexanesulphonate and acetate buffer In this way chromatograms with high resolution and good peak symmetry for the HAs studied were obtained Typical chromatograms obtained with different mobile compositions are given in Figs 6 and 7 The HAs are eluted in different orders depending on the chromatographic conditions The system peaks S_1, S_2 and S_3 are due to copper ion, acetate and hexanesulphonate respectively Peak S_1 is negative in all the chromatograms Peak S_2 disappears gradually with decreasing concentration of acetate buffer Peak S_3 is positive or negative depending on the concentration of hexanesulphonate At pH < 4 5 peak S_3 is not eluted within 30 min

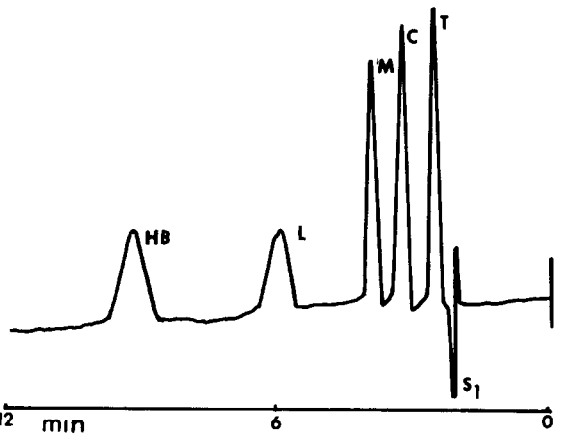

Fig 6 Chromatogram of a mixture of HAs Mobile phase, 0 5 m*M* Cu(II)–5 m*M* hexanesulphonate–2 m*M* acetate buffer (pH 4 2), flow-rate 1 ml/min, detection, UV at 254 nm, 0 08 a u f s , temperature, 30°C, concentration of samples, 0 1–1 m*M* Peaks S_1 = system peak, C = CA, T = TA, M = MA L = LA, HB = HBA

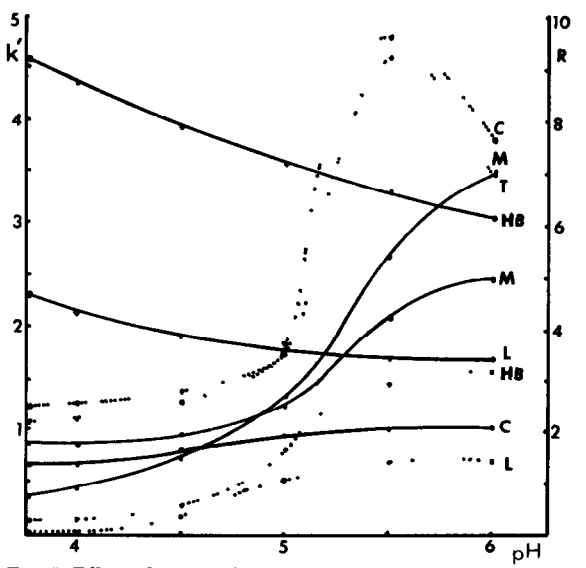

Fig 5 Effect of pH on the retention and detection response of HAs Conditions as in Fig 2 except 0 5 m*M* Cu(II) and pH 5 6 Lines as in Fig 2

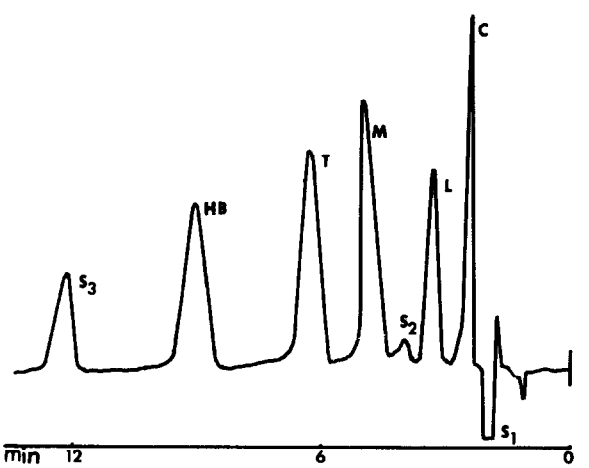

Fig 7 Chromatogram of a mixture of the HAs Mobile phase, 0 5 m*M* Cu(II)–5 m*M* hexanesulphonate–5 m*M* acetate buffer (pH 5 6), detection, UV at 254 nm, 0 16 a u f s , concentration of HAs, 0 1–0 5 m*M* Peaks S_1, S_2, S_3 = system peaks Other conditions and peaks as in Fig 6

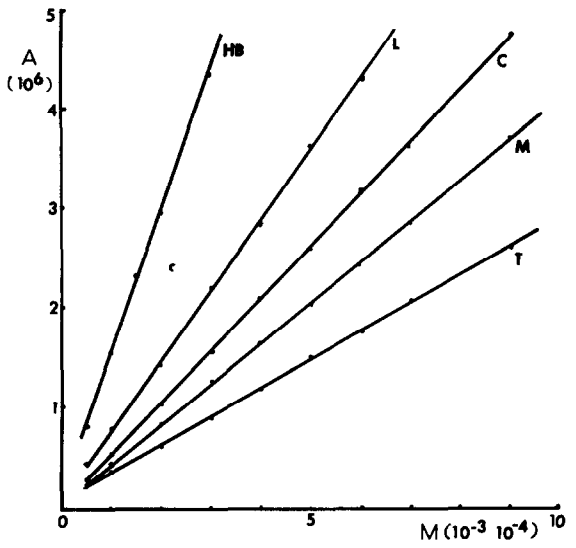

Fig 8 Calibration graphs of integral peak area values (A) *versus* HA concentrations injected Concentration coordinate is $\times 10^{-3}$ M for HBA and LA and $\times 10^{-4}$ M for CA, MA and TA Chromatographic conditions as in Fig 7

Determination of hydroxy acids

If the mobile phase conditions are optimized and held constant, determination of the HAs is possible with Cu(II)-aided UV detection Calibration graphs were obtained for HAs in the range of sample amounts from 10^{-6} to 10^{-4} mmol under the same conditions as in Fig 7 No attempt was made to determine linearity above 10^{-4} mmol Typical calibration graphs of integral peak areas *versus* the sample concentration injected are shown in Fig 8 Owing to the background absorbance of the mobile phase and the background noise of the instrument, the calibration graphs do not pass through the origin The graphs can be fitted to a linear regression equation

$$A_i = a + bm_i \quad (3)$$

where A is the integral peak area value, a is the intercept, b is the slope and m is solute amount The slopes should be related to the detection sensitivity of the HAs, which may be defined as the change in response intensity per unit change in solute amount, that is

$$S = \Delta R/\Delta m_i \quad (4)$$

With a UV detector for concentration-sensitive detection, the sensitivity is given in units of absorbance $\times$ ml/mM and can be calculated from the absorbance corresponding to full-scale deflection of the recorder at maximum detector sensitivity Table II gives the intercepts a, slopes b, correlation coefficients r and detection sensitivity S for the HAs The correlation coefficients are over 0 99 for all the samples, showing good linearity of the calibrations

Minimum detectable amount

The minimum detectable amount is defined as the amount of solute that produces a response of the chromatographic peak height equal to three times the noise level The minimum detectable amounts of the HAs under the chromatographic conditions used were calculated and are given in Table II Most of them are below 1 nmol and hence trace analyses for the HAs are practicable

TABLE II

DATA FOR DETERMINATION, SENSITIVITY AND MINIMUM DETECTABLE AMOUNT

HA	Intercept, a[a]	Slope, b[a]	Correlation coefficient, r	Sensitivity, S (absorbance ml/mM)	Q_{min}[b] (mol)
LA	30	3 6 10^6	0 9972	464	4 2 10^{-10}
BHA	15	7 3 10^6	0 9989	928	2 5 10^{-10}
CA	30	2 8 10^7	0 9993	3520	7 9 10^{-11}
MA	20	2 1 10^7	0 9981	2720	6 3 10^{-11}
TA	17	1 3 10^7	0 9967	2240	3 5 10^{-10}

[a] Integral values
[b] Minimum detectable amount

CONCLUSIONS

The proposed method allows the separation and determination of multi-functional HAs by reversed-phase chromatography with the aid of copper ion in the mobile phase containing acetate buffer and hexanesulphonate The dynamic ion exchange and ion-pair partition of HA–Cu(II) complexes formed in the chromatographic process can offer significant advantages over fixed-site ion exchangers of a bonded phase for the separation of ion interaction and hydrophobicity and also for detection sensitivity of the HAs Detection is performed at 254 nm, avoiding interferences that occur at lower wavelengths The analysis is simple and rapid Also, the sensitivity, accuracy and precision appear to be comparable to those in alternative methods

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