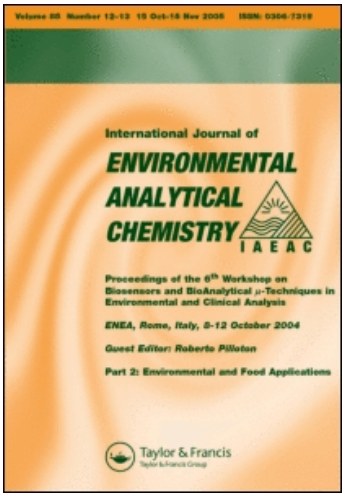

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Retention and Spectral Characteristics of Copper-Natural Ligand Complexes in Reversed Phase C18-HPLC with UV-PDA and Fluorescence Detection

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RETENTION AND SPECTRAL CHARACTERISTICS OF COPPER-NATURAL LIGAND COMPLEXES IN REVERSED PHASE C18-HPLC WITH UV-PDA AND FLUORESCENCE DETECTION

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Copper complexes with fulvic (FA), caffeic (CFCA), vanillic (VA) and salicylic (SA) acids were investigated by reversed-phase HPLC with dual uv-vis photodiode array (PDA) and fluorescence (fl) detection using retention enrichment (RE) and direct complex introduction (DCI) methods. In the RE method VA and FA formed a hydrophobic Cu(II)-complex with measurable shift in retention time (t_R). Whether or not there was a shift in t_R, the uv-vis spectral characteristics of all complexed ligands in the RE method were of reduced absorption intensities but with the same characteristic absorption bands. In the DCI method only CFCA formed a more hydrophobic Cu(II)-complex with a measurable shift in t_R and a distinguishable uv-vis spectrum. Free FA was resolved into seven uv-vis absorbing and fluorescing peaks (binding sites). At molar ratio M:L of 1:1 and pH 7, Cu-FA complex was resolved into the same peaks but with less uv and fluorescence intensities. The complexed peaks contained 69% of the added Cu(II) and total Cu recovery was 87%. Conditional stability constants calculated from the fl or uv spectra were comparable.

KEY WORDS: Metal binding, equilibrium constants, humic substances.

INTRODUCTION

In recent years the use of RP-HPLC, for quantitative evaluation of equilibrium constants for metal bindings by organic ligands or for analysis of organometallic complexes, has dramatically increased[1-8]. The measurement of retention value in RP-HPLC under appropriate conditions should give quantitative information on certain physico-chemical phenomena which take place in solution. One approach for measuring the stability constants of metal–ligand complexes, involves the enrichment of retention of a ligand in the presence of the metal ion in the mobile phase[5-8]. Horvath and co-workers[5,7,8] developed a relationship between capacity factor k and the conditional stability constants K for a number of organic

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ligands and metal ions. Several association constants for metal binding by various nucleotides were successfully measured by RP-HPLC.

Another approach involves the direct complex introduction (DCI) to the HPLC system. In general it has been possible to separate a number of metal complexes in a single chromatographic determination. However problems could arise due to the labile nature of the complex or the weak formation constant of the metal complex or due to the presence of metal parts in the chromatographic system or the ion exchange properties of the silica support[9–13]. In natural waters metal organic complexes play an important role in the transport and availability of toxic metals. Considerable amount of information is available in the literature on the stability constants of Cu-humate complexes and chelates[14–17], or on humate-metal interaction models[18–24], but relatively little is known about the overall molecular description of the complexes. This is mainly due to the complexity and heterogeneity of aquatic humic material. Only few publications have appeared in the literature on the use of HPLC to study fulvic acids (FA)-metal complexes. Mantoura and Riley[13,15] used the gel filtration method to show that FA from lake water or peat each has two binding sites. In other research the technique was essentially used to concentrate metal-FA complexes from surface or marine waters. In natural waters it was found that most of the complexed Cu (II) were of intermediate polarities[25,26]. Several Cu-organic complexes of high and intermediate polarities were separated from sea water[27]. Application of RP-HPLC to study metals interactions with polyfunctional natural ligands such as CFCA, VA, SA, and FA offer several attractive possibilities, however the system should first be carefully evaluated. Important questions relevant to the use of RP-HPLC to study natural organic ligands-metal complexes can be formulated as follows; i) how the retention and spectral characteristics of the ligand and complexed ligand differ under the RE method or DCI method? ii) is it possible to efficiently separate the free ligand from the complexed species? iii) what is the mass balance of the metal in a typical complex chromatogram and iv) what are the influences of the M:L molar ratio, and the kinetic factors on the chromatograms? This paper presents the results of an investigation designed to address these questions.

EXPERIMENTAL

The approach in this research was to i) utilize the separation equilibria in RP-HPLC to study the retention behavior of a number of natural organic ligands and their Cu(II) complexes, ii) simultaneously use UV-PDA, fluorescence and AA to characterize the separated peaks, iii) study the influence of reaction time on the complexation reactions.

Materials

References Suwannee River FA was purchased from the International Humic Substances Society[28]. Model compounds included caffeic acid, vanillic acid and salicylic acid were all ACS high purity chemicals. Solutions of Cu (II) were prepared from $CuSO_4.5H_2O$ or $CuNO_3.3H_2O$ crystals. All other reagents were ACS high purity chemicals. Pure HPLC water was obtained from the milli-Q system and the organic solvents were all HPLC grades.

Instrumentation

1-Fluorescence Scanning; Aminco spectrofluorometer model 4–8202 equipped with a ratio photometer. 2-Hewlett Packard HPLC Model 1090 with UV-VIS Photodiode Array (PDA) and Schoeffel Model 970 Fluorescence detectors; 3-Perkin Elmer model 2380 atomic absorption spectrophotometer. The RP-HPLC columns included; Boundclone C18, (phenomenex) 300 mm 1 X 39 mm id; Hypersil ODS C18 200 mm 1 X 2.1 i.d. mm and Nova-Pak C18 150 mm 1 X 4 mm id.

Methods

An outline of all the experiments is shown in Figure 1. Under isocratic conditions the RE method was applied using mobile phases consisting of 0.01 M acetate buffer and 0.01 M Na_2SO_4 or $CuSO_4$. The mobile phases composition and the HPLC operating conditions for the RE method are shown in Table 1. solutions of ligands were prepared in methanol at concentration $1X10^{-4}$M. Each sample was run with (O) and without (X) the presence of Cu^{2+} in the mobile phase. In the gradient DCI experiments, complexation experiments were done outside the HPLC instrument and aliquots of free and complexed sample solutions were introduced to the HPLC system under the same conditions. Mobile phases composition and the gradient programs used in the DCI method are shown in Table 2. CFCA was solubilized in phosphate buffer at pH 7 at concentration of $1X10^{-4}$M. The solution was equilibrated with Cu^{2+} at M:L ratios of 1:1, 1:2 and 1:4. Aliquots of free and complexed CFCA acid were analyzed by HPLC at 24, 48, 96 and 144 hours from the time of preparation. The preparative experiments were run only the 24 hours equilibrated samples. Fractions were collected from all regions of the chromatogram. Collected fractions were freeze dried to the original volume of the sample and subjected to Cu analysis by AA and fluorescence scanning. Fulvic acid complexation experiment was done the same except that only one molar ratio of 1:1 was used. Several analytical quality control steps were followed throughout this research. These included the following: (a) each sample was injected in triplicates into the HPLC system; (b) with each mobile phase, after establishing the base line a procedure blank is injected before each sample; (c) all samples were filtered through 0.45 um glass fiber filter before injection into the HPLC system.

Chromatographic data interpretation

a) In the RE method, t_R and the unretained solvent time (t_{RO}) were used to calculate the capacity factors (k) for the peaks, using the uv and/or the fl signals. The t_R was measured in mm from zero t_R time to the peak maximum and converted to minutes. The dead volume time t_{RO} was measured in triplicates by the NO_3 method[29]. Each sample was injected least three times and the reproducibility was better than 95 percent.

b) In the DCI method both the uncomplexed and complexed samples were injected into the

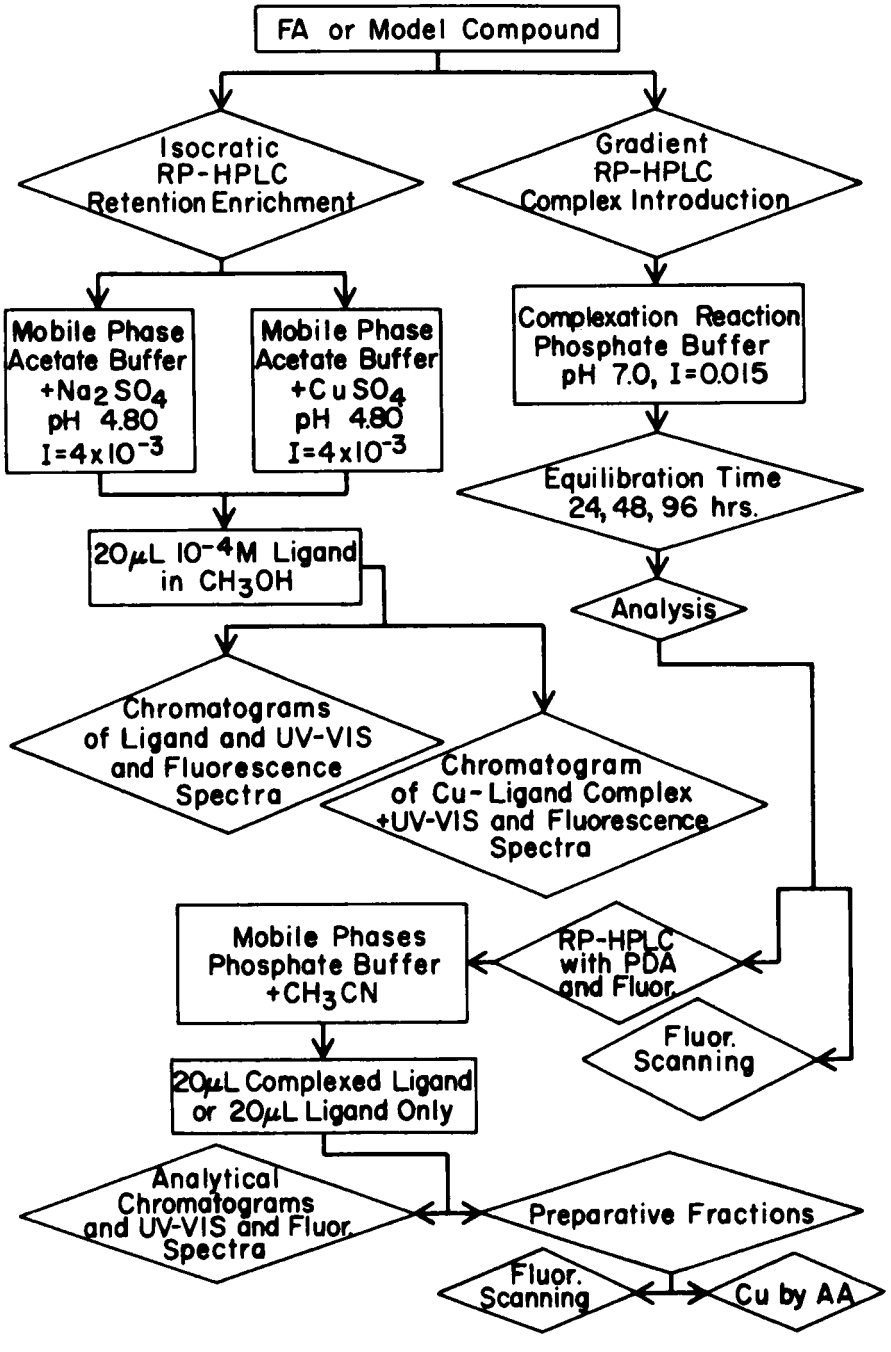

Figure 1 Experimental outline.

Table 1 RP-HPLC experimental conditions used in the RE method.

Column: Boundclone 10 C18 (Phenomenex) 10 um particle size; 30 cm 1.×0.45 cm i.d.)

Mobile Phases:

(X) Without Cu^{2+}: 85 % aqueous 1.0×10^{-3} M acetate buffer, 1.33×10^{-3} M Na_2SO_4, pH 4.80 and 15 % CH_3CN.

(O) With Cu^{2+}: 85% 1.0×10^{-3} M acetate buffer, 1.0×10^{-3} M $CuSO_4$, pH 4.80 and 15% CH_3CN.

Samples: 1.0 X 10^{-4} M CFCA, VA, SA, and FA in CH_3OH

Sample Loop: 50 ul

Flow Rate: 0.30 ml/min

Detectors:

UV-VIS-PDA; *Chromatograms monitored at* λ 254 *nm*

Fluorescence with	λ_{ex}	and	λ_{em} nm
CFCA	350		470
VA	380		418
SA	300		418
FA	375		470

Table 2 Typical gradient programs used in the DCI method.

gradient program	*mobile phase*	*pH at 25°C*	*gradient*	*%A*	*%B*	*column types*	*samples*
I	A: H_20 + 500 fold diluted phosphate buffer B: CH_3CN	5.6	t0 min	98	2	Hypersil ODS C18 100 mm length × 2.1 mm i.d., flow = 0.5 ml/min	(4:1) CFCA/Cu soln at 24, 48, 96, and 144 hrs
			t2 min	95	5		
			t5 min	70	30		
			t7 min	15	85		
			t10 min	15	85		
			t12 min	70	30		
			t15 min	98	2		
			t20 min	98	2		
II	A: H_20 + 500 fold diluted phosphate buffer B: CH_3CN	4.8	t0 min	99.8	0.2	Hypersil ODS C18, 100 mm length × 2.1 mm i.d., flow = 0.5 ml/min	(1:1), (2:1), and (4:1) CFCA/ Cu at 24 and 48 hrs, (4:1) ADPA/ Cu at 24 hrs, and (1:1) FA/Cu solns
			t1 min	99	1		
			t2 min	70	30		
			t4 min	10	90		
			t8 min	5	95		
			t10 min	5	95		
			t12 min	10	90		
			t15 min	40	60		
			t18 min	99.8	0.2		
			t20 min	99.8	0.2		
III	A: H_20 + .01% AcH B: CH_3CN	2.9	t0 min	99	1	NOVA-PAK C18 150 mm length× 4 mm i.d. flow = 0.2 ml/min	(1:1) FA/Cu soln
			t2 min	70	30		
			t4 min	40	60		
			t10 min	15	85		
			t15 min	15	85		
			t20 min	99	1		
			t25 min	99	1		

HPLC instrument under the same mobile phase and Instrumental conditions. The separation was simultaneously monitored by the uv-PDA and fl detectors. The stability constant of each peak was calculated from both the uv and fl chromatograms. The peak areas were measured by the cut and weigh method. An electronic balance sensitive to five decimal places was used for weighing the cut peaks. The degree of fluorescence quenching (x) is used as a parameter for determination of the conditional stability constant K of the resolved peaks. The area of the peaks (unquenched I_{max}), partially quenched (I) and totally quenched (I_{min}) for a specific fraction is value of x for the binding site or ligand is obtained. The same approach was applied to the uv chromatograms.

$$x = \frac{I_{max} - I}{I_{max} - i_{min}} \tag{1}$$

For a single binding site, the conditional stability constant Kn defined as;

$$K_n = \frac{[CuLn]}{[CU]\,[L]^n} \tag{2}$$

For ML and ML_4 models, n=1 and n=4 were used.

$$[CuL_n] = \frac{x\,L_t}{n} \tag{3}$$

$$[Cu] = Cu_t - \frac{xL_t}{n} \tag{4}$$

$$[L] = (1-x)\,L_t \tag{5}$$

For multiple binding sites such as FA the fraction for the i-th binding site Y<i> was defined as;

$$Y\langle i\rangle = \frac{L\langle i\rangle}{L_t} \tag{6}$$

$$L_t = \Sigma^m\, i{=}1\; L\langle i\rangle \tag{7}$$

Where m= the number of binding sites or peaks.

The fraction Y <i> is assumed to be;

$$Y\langle i\rangle = \frac{A\langle i\rangle}{A_t} \tag{8}$$

$$A_t = \Sigma^m\, i{=}1\; A\langle i\rangle \tag{9}$$

$$K_n \langle i \rangle = \frac{[CuL \langle I \rangle_n]}{[Cu]\,[L \langle i \rangle]^n} \tag{10}$$

$$[CuL \langle i \rangle_n] = \frac{x \langle i \rangle\, Y \langle i \rangle\, L_t}{n} \tag{11}$$

$$[Cu] = Cu_t - \Sigma^m{}_{i=1} \frac{x \langle i \rangle\, Y \langle i \rangle\, L_t}{n} \tag{12}$$

$$[L \langle i \rangle\,] = [1 - x \langle i \rangle\,]\, Y \langle i \rangle\, L_t \tag{13}$$

List of Abbreviations:

$[CuL_n]$ = Concentration of the complex.
$[Cu]$ = Concentration of the Cu ion.
$[L]$ = Concentration of the free ligand.
n = Coordination number for the complex.
L_t = Total ligand concentration.
$L\langle i \rangle$ = Ligand concentration for the i-th binding site.
$Y\langle i \rangle$ = Fraction of the i-th binding site.
m = Number of binding sites or resolved peaks.
A_t = Total areas of all the binding sites or peaks.
$A\langle i \rangle$ = Peak area for the i-th binding site.

Conditional stability constants data were derived from; i) fluorescence quenching in the total sample or ii) the fluorescence or uv responses of the fractions in the HPLC chromatograms. and iii) AA analysis of Cu in the preparative fractions. Methods of calculations of stability constants are based on published methods[30,31].

RESULTS

The RE Method, isocratic RP-HPLC chromatograms

Figure 2-A shows the 254 nm uv chromatograms of the investigated compounds, with (O) and without (X) the presence of Cu^{2+} in the mobile phase. The corresponding uv-vis scans of the peaks are shown in Figure 2-B and the corresponding fluorescence chromatograms are shown in Figure 2-C. Table 3 lists the capacity factors (k) for the free and complexed ligands and the amount of change in the k values due to complexation with Cu^{2+}. For CFCA pK_{a1} is 4.45 which is close to the pH of the mobile phase (4.80). Thus both dissociated and undissociated acid species would be of equal concentrations[32]. The uv chromatogram of the free CFCA in Figure 2-A shows almost the same peak area for the dissociated CFCA peak

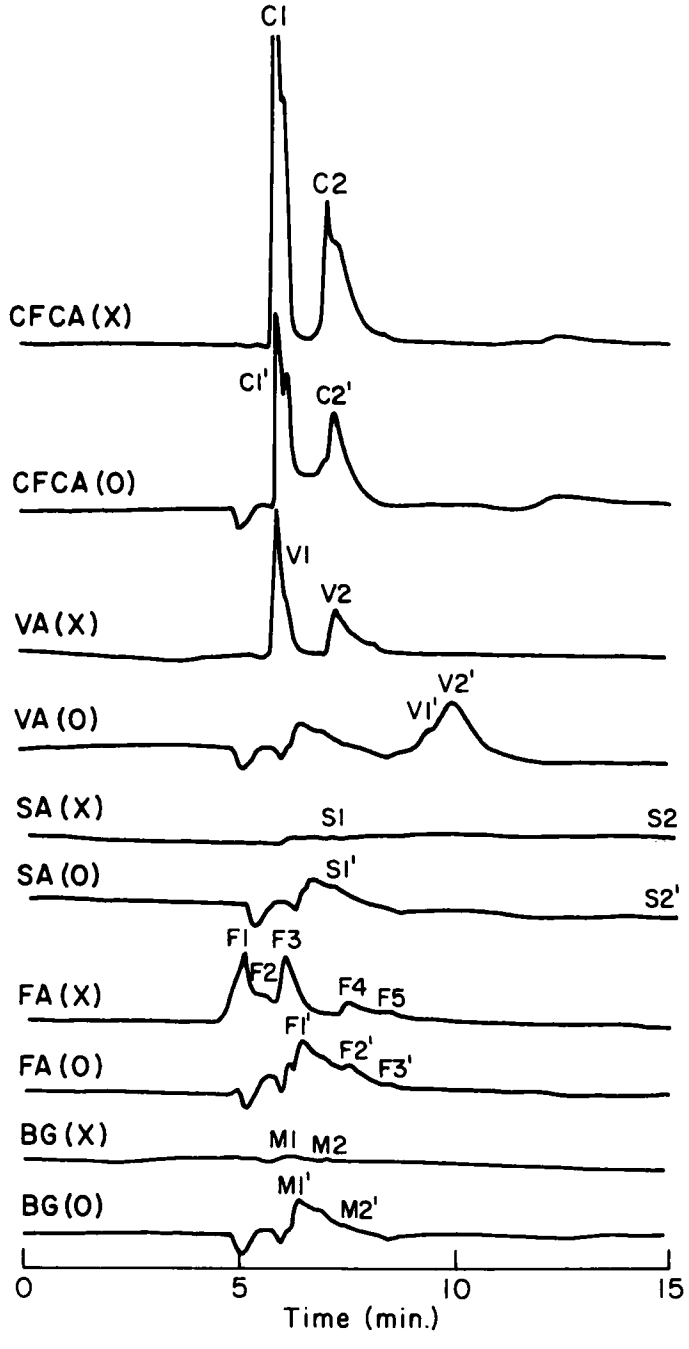

Figure 2-A Chromatogram (uv 254 nm) of 50 µl 10^{-4} M CFCA, VA, SA, and FA in methanol. Experimental conditions are listed in Table 1, (O) mobile phase with Cu^{2+} and (X) mobile phase without Cu^{2+}.

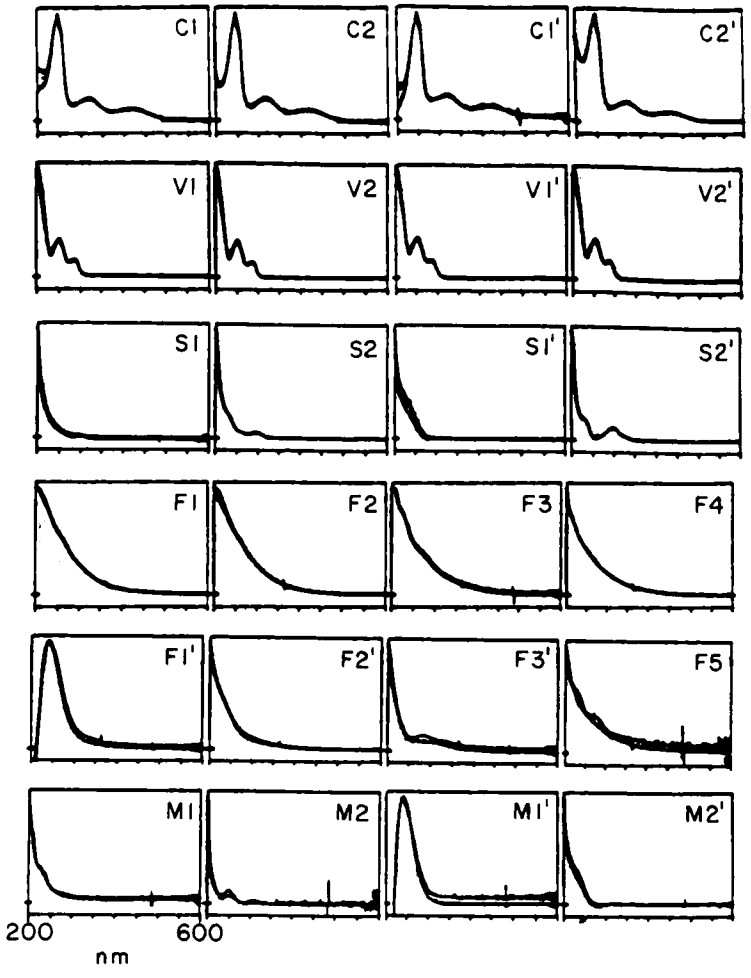

Figure 2-B Uv-vis scans of the peaks shown in Figure 2-A. Experimental conditions are shown in Table 1.

C1 at k = 0.24 and the undissociated CFCA peak C2 at k = 0.53. In the Cu-CFCA chromatogram both peak show minor shifts (Δ k = <0.15), due to the presence of Cu^{2+} in the mobile phase. The uv-vis scans of both CFCA and Cu-CFCA show three major absorption bands at λ248 nm, 318 nm and 415 nm. The scans are different from those published[33] or found in the DCI method. This is possibly due to the solvent effect (0.01 M acetate buffer and 0.01 M Na_2SO_4) on the uv-vis spectra of CFCA. This solvent effect seems to be more pronounced in the fl chromatograms as shown in Figure 2-C where a very weak signal is noted for the free CFCA and no signal is detectable in presence of Cu^{2+}. This is in contrast to what is found under gradient RP-HPLC with phosphate buffer-acetonitrile in the DCI method.

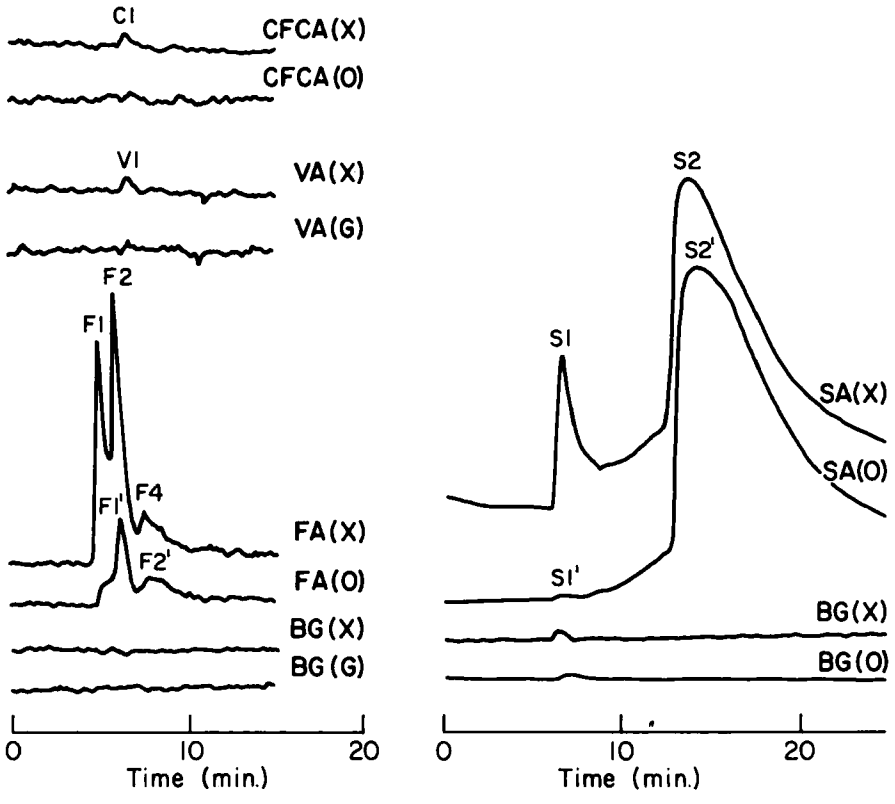

Figure 2-C Fluorescence chromatogram of 50 ul 10^{-4} M of CFCA, VA, SA, and FA in methanol. Experimental conditions listed in Table 1, (O) mobile phase with Cu^{2+} and (X) mobile phase without Cu^{2+}.

For VA the chromatogram in Figure 2-A shows the same peak area for dissociated peak V1 at k = 0.29 and the undissociated peak V2 at k = 0.82. This is also due to the close values of the pk_{a1} 4.36 of VA, and the pH 4.80 of the mobile phase. The Cu-VA chromatogram shows a major shift in the t_R (Δ k = 0.82) of the peak V1. However the undissociated peak V2 showed less shift in t_R with (Δ k = 0.40). The measurable shift in t_R in the presence of Cu^{2+} in the mobile phase, is attributed to formation of a more hydrophobic complexed VA. The uv-vis scans of all the major free VA peaks show three major absorption bands at λ205 nm, 250 nm and 285 nm. The spectra are similar to those published for VA[33]. The uv-vis scans of the Cu-VA peaks showed reduced absorption intensities but the same absorption bands. For structural reasons VA is likely to act as a monodentate ligand and thus complexation could cause changes in the molecular surface properties of VA, resulting in increased t_R. The reduced uv intensity can be attributed to the blue shift resulting from Cu^{2+} ion tying up the electrons on the ligand's functional groups in VA. As found in case of CFCA the fluorescence signals of VA with (O) and without (X) Cu^{2+} are very weak possibly due to the solvent effect.

Table 3 Capacity factors $K^{(1)}$ for CFCA, VA, SA and FA and their Cu complexes in the RE method.

Mobile Phase (X) Without Cu^{2+}	*Mobile Phase (O) With Cu^{2+}*	*Change in k* Δk
$K_{C1} = 0.24$	$K_{C1'} = 0.31$	0.07
$K_{C2} = 0.53$	$K_{C2'} = 0.60$	0.07
$K_{V1} = 0.29$	$K_{V1'} = 1.11$	0.82
$K_{V2} = 0.82$	$K_{V2'} = 1.22$	0.40
$K_{S1} = 0.56$	$K_{S1'} = 0.60$	0.04
$K_{S2} = 2.18$	$K_{S2'} = 2.29$	0.11
$K_{F1} = 0.09$	$K_{F1'} = 0.40$	0.31
$K_{F2} = 0.22$	$K_{F2'} = 0.64$	0.42
$K_{F3} = 0.31$	$K_{F3'} = 0.84$	0.53
$K_{F4} = 0.84$	—	—
$K_{F5} = 1.09$	—	—
$K_{M1} = 0.38$	$K_{M1'} = 0.62$	0.24
$K_{M1} = 0.56$	$K_{M1'} = 0.67$	0.11

(1) $K = t_R - t_O/t_O$; $t_O = 4.5$ min. (determined by the NO_3^- (29))
t_R = retention time in minutes, measured in mm from zero time to peak maximum then converted to minutes.
Each sample was run in triplicates and reproducibility of t_R measurements were better than 95%.
Chromatograms are illustrated in Figures 2-A and 2-C.

For SA, pK_{a1} is 2.98 and is quiet different from the pH of the mobile phase 4.80 and thus only one peak representing the dissociated acid is expected. For the uncomplexed SA the 254 nm chromatograms showed a very weak signal at 7.5 to 8.0 minutes. The chromatogram of Cu-SA shows also a weak signal and a minor shift in the t_R (Δ k = 0.04). However the corresponding free SA fluorescence chromatogram shows a more distinct peak at t_R 7.5–8.0 min. and a major ghost peak at t_R between 14 to 20 minutes. The fluorescence chromatogram for the Cu-SA shows almost complete quenching of the first peak S_1 but enhancement of the ghost peak S_2. Fluorescence quenching can be attributed to enhanced intersystem crossing by the paramagnetic Cu^{2+}. The uv-vis scans of the ghost peaks S_2 and S_2' are very weak indicating that the fluorescence responses are not directly related to the molecular excitation of SA at λ_{ex} 300 nm. The ghost peak is likely due to the contamination present in SA.

The uv 254 nm chromatogram of free FA shows a poor resolution of 5 areas of response for the free FA. The Cu-FA chromatogram show two negative peaks similar to those present in the background and three poorly resolved peaks with shift in k values ranging from 0.31 to 0.53. Peak assignments are inconclusive due to the difficulty in following peak identities in presence of Cu^{2+} in the mobile phase. The uv-vis scans of the major peaks in the free FA chromatogram shows featureless spectra with no or few characteristic absorption bands. The spectra are similar to those reported for total FA or its fractions[36–39]. The strong uv absorption of peak F′ is similar to M1′ in the background and is likely due to Cu-CH_3OH complex. The scans of Cu-FA peak F3′ shows a minor absorption band at λ 365 nm due to the charge transfer band characteristic to catechol type bonding[40]. The fluorescence chromatogram of free FA shows three poorly resolved regions of emission. For the complexed FA only two regions are detectable. Because of the modest quality of FA separation no further experiments were made using the RE method.

In summary, application of the RE method provided useful information on the retention and spectral characteristics of the investigated compounds. Horvath *et al.*[5,7,8] discussed changes occurring in the molecular properties of a solute upon complexation with metal ions. In most cases it suffies to consider one or more of the following alterations in the solute properties; i) As a result of complexation, the molecular surface area of the solute changes and thereby the contact area of the complex, upon binding to the non-polar surface of the stationary phase will be different from the corresponding contact area of the uncomplexed solute. ii) Upon complexation the dipole moment of the solute becomes greater or smaller than that of the free solute. iii) The process produces an alteration of the net electronic charge of the solute molecule. Of the compounds investigated in this paper, VA and FA showed measurable shift in t_R. To determine Cu-VA or Cu-FA stability constants, several mobile phases with various concentrations of Cu^{2+} are necessary. There is a potential of using the RE method with FA if better separation conditions can be developed. In case of CFCA and SA the slight change in k (Δ 0.15) indicates minor changes in the molecular surface areas and the polarities of these ligands upon complexation.

For all of the investigated compounds the characteristics spectra of the free and complexed species were indistinguishable in relation to the position of absorption bands or the appearance of new bands (within the range of λ 200–600 nm). The concurrent reduction or quenching of the uv absorption or fl emission can be attributed to the Cu^{2+} tying up the outer electrons of the ligand's functional groups. This would result in reduction of both the uv and fl signals. Fluorescence quenching is also attributed to enhanced intersystem crossing by the paramagnetic Cu^{2+}[34,35]. Precipitation of the Cu-complex is another possibility but can be excluded due to the high Cu:L in the RE method. The charge transfer band in the range of λ 365–420 nm which is characteristic of Cu(II)-phenolate interaction was barely detectable in one of the scans in Cu-FA peaks (F3′). Maintaining M:L of 10:1, ensures the formation of Cu complex during the elution process and thus data on both the retention and spectral characteristics reflect those of the copper complexed species. The use of acetate buffer-sodium sulfate in the mobile phase dramatically reduced the responses from the PDA or the fluorescence detectors and in case of free CFCA the characteristic bands were altered.

The DCI method; gradient RP-HPLC chromatograms CFCA and CFCA/Cu solutions:

Figure 3-A shows the 254 nm uv chromatogram and the uv-vis scans of CFCA at 24,48,96 and 144 hrs after preparation, using phosphate buffer gradient program I. In contrast to the RE method the CFCA chromatogram shows a single major peak at t_R 3.5–5.0 min. with about one min. shift in t_R after 144 hr. Detection of a single peak is mainly due to the higher pH (5.60) of the phosphate buffer mobile phase in comparison to the acetate buffer at pH 4.80. It is noted that the major peaks labeled 1, 1′, 1″ and 1* show well defined bands and high purity as indicated by the uv-vis scans. The spectra are characteristic of CFCA with three major distinct absorption bands at λ215, 285 nm and 310 nm. The spectra are the same as those published[33], but are different from those detected for CFCA by the RE method. It is also noted that the area under the CFCA peak tends to decreases with time. This was associated with a one minute shift of the t_R of the major peak and the appearance of an

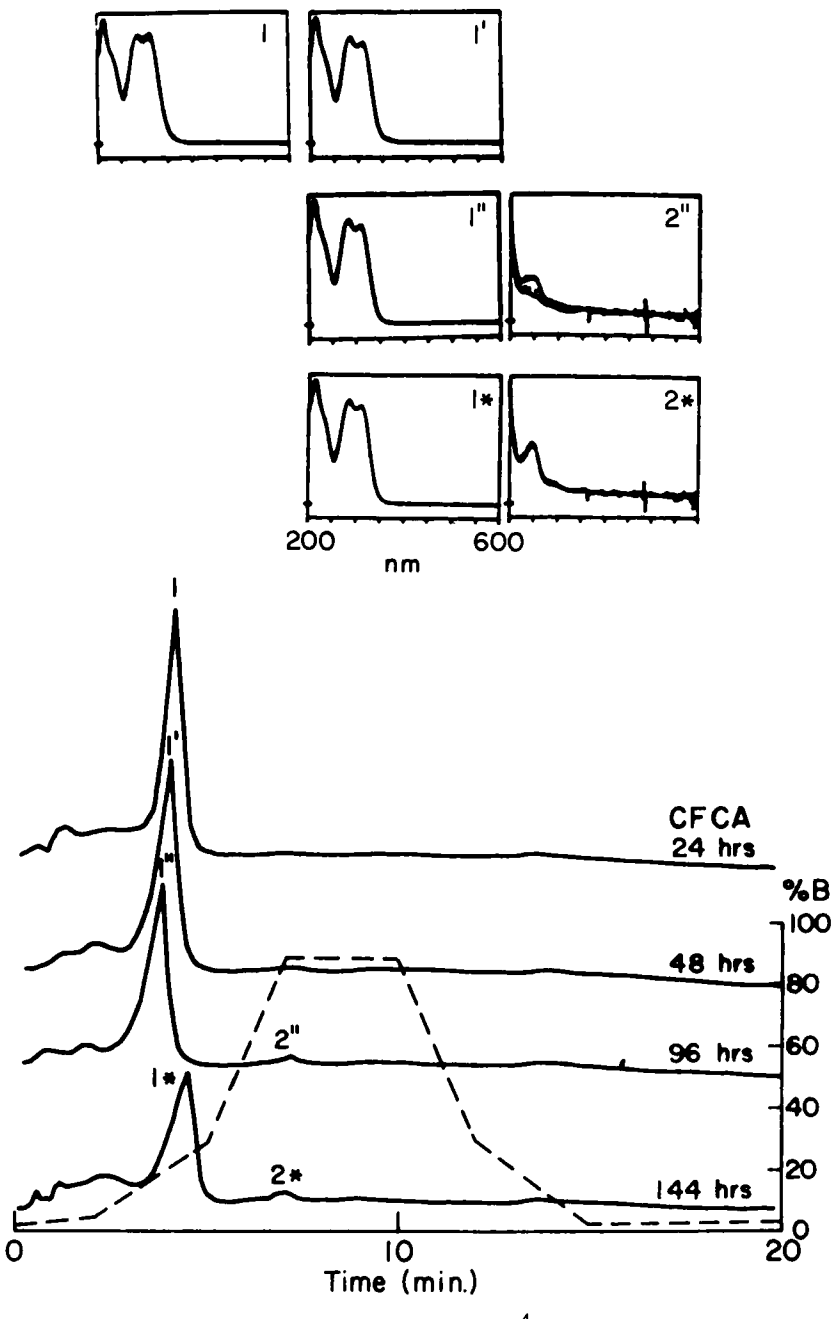

Figure 3-A Chromatogram (254 nm) and uv-vis scans of 20 ul 5.50×10^{-4} M free CFCA used in the DCI method. Experimental conditions are shown in Table 2, column Hypersil ODS C18, gradient program I.

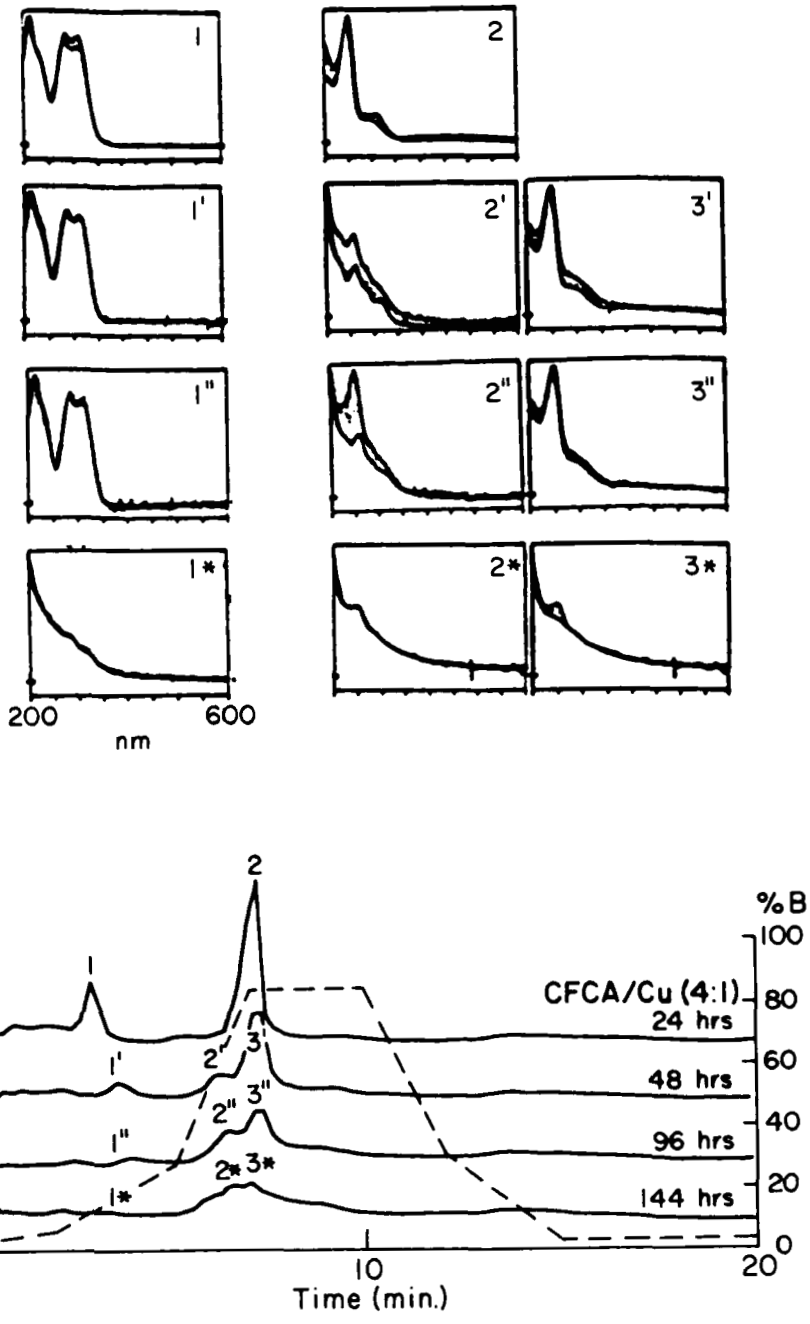

Figure 3-B Chromatogram (254 nm) and uv-vis scans of 20 ul 5.55×10^{-4} M Cu-CFCA (1:4) used in the DCI method. Experimental conditions are shown in Table 2, column Hypersil ODS C18, gradient program I.

additional peak 2″ at longer t_R indicating the instability of CFCA solution in phosphate buffer. Solution instability was also observed in the color change and precipitation after 144 hrs from preparation. All these change are attributed to CFCA polymerization. Figure 3-B shows the 254 chromatogram and uv-vis scans of Cu-CFCA (1:4) at 24, 48, 96 and 144 hrs using the same gradient program I. The first peaks labelled 1, 1′ & 1″ represent unreacted CFCA and exhibit the characteristic uv spectrum of CFCA. The peaks occurring at 6–8 minutes, and labelled 2′2′;3,3″ exhibit a different spectrum with two characteristic absorption bands at λ246 nm and 308 nm. The influence of complexation time on the chromatograms is similar to what was found for free CFCA. The color change and precipitation of the Cu-CFCA solution seemed to be faster than in the free CFCA solution.

Results of the Cu recovery experiments are shown in Table 4. Total Cu recoveries from the HPLC system ranged from 83.9 to 87.6%. It is noted that peak 1 for the 1:4 Cu-CFCA, 24 hr, in figure 3-B, contains 0.1–0.2 ug Cu (II). Peak 2 in the same chromatogram contains 1.6 ug Cu(II). For the 1:2 and 1:1 24 hr experiments (chromatograms are not shown, but were comparable to those of 1:4 experiment) the Cu(II) content of peak 2 were 2.2 and 2.90 μg, respectively. These results present adequate evidence to conclude that peak 2 in the 24 hr recovery experiments, corresponds to Cu-CFCA complex. It is also noted that the Cu content of peak (2) decreases as the L:M ratio decreases and its value coincides with the x values derived from the chromatograms. The increased concentration of Cu^{2+} in background II is due to the wash out of the ionic Cu^{2+} as the concentration of the phosphate increases from 30% at t_R 12 min. to 98% at t_R 20 min. (Program I in Table 2).

Conditional stability constants of Cu-CFCA complex calculated from fluorescence scanning of the total samples, or from HPLC uv or fluorescence quenching are shown in

Table 4 Elemental Cu content in preparative HPLC fractions, determined by AA (Results in ug Cu).

		Total Cu	*Peak 1*	*Peak 2*	*Background I*[(a)]	*Background II*[(b)]	*Recovery %*
Cu/CFCA							
(1:1)		36.80	0.10	2.60	6.40	23.60	88.85
		34.20	0.10	3.20	7.80	18.40	86.26
	mean	35.50	0.1	2.9	7.1	21.0	87.6
(1:2)		18.80	0.10	2.10	3.30	11.00	87.76
		17.20	0.10	2.30	2.90	9.00	83.14
	mean	18.0	0.1	2.2	3.1	10.0	85.5
(:41)		8.40	0.20[(c)]	1.8[(c)]	0.90	4.30	85.71
		9.00	0.20	1.40	0.70	5.10	82.22
	mean	8.7	0.2	1.6	0.8	4.7	83.9
Cu/FA							
(1:1)		3.70	1.80[(d)]	1.20[(e)]	0.40	0.00	91.89
		4.10	1.40	1.00	1.00	0.00	82.92
	mean	3.9	1.6	1.1	0.7	0.0	87.2

(a) Background I corresponds to fraction representing base line eluent.
(b) Background II corresponds to fraction representing base line post run eluent.
(c) Fractions representing peak 1 or 2 in Figure 3-B for the 24 hr. 4:1 complex.
(d) Fraction represents peaks 2–6 in Figure 4-B.
(e) Fraction represents peak 7 in Figure 4-B.

Table 5 Conditional stability constants for Cu-CFCA from total fluorescence, RP-HPLC and preparative elemental Cu.

Total Fluor. Scanning	*RP-HPLC-Fl*	*RP-HPLC-UV*	*Prep. AA*
(1:1) x = 0.90	0.64	0.70	Cu = 2.90 ug
$K_1 = 1.55\times10^5$	8.70×10^3	1.43×10^4	1.79×10^2
$K_4 = 2.85\times10^{16}$	1.16×10^{14}	2.93×10^{14}	$4.9\,6\times10^{12}$
(1:2) x = 0.88	0.57	0.67	Cu = 2.2 ug
$K_4 = 3.8\,9\times10^{16}$	1.36×10^{14}	4.43×10^{14}	5.0×10^{12}
(1:4) x = 0.84	0.50	0.61	Cu = 1.6 ug
$K_4 = 9.10\times10^{16}$	1.77×10^{14}	$6.99\,10^{14}$	5.5×10^{12}

The solutions of (1:1) Cu = 5.55×10^{-4} M, (1:2) Cu = 2.78×10^{-4} M and 1:4 Cu = 1.39×10^{-4} M with CFCA = 5.55×10^{-4} M in pH phosphate buffer/methanol, pH 4.8, uv λ 254 mm, fl λ_{ex} 350 mm λ_{em} 470 mm, K_1 is based on 1:1 model CuL and K_4 is based on 1:4 model CuL_4.

Table 5. The table shows that the numerical values of K derived from the experimental data, though reproducible by any one single method, can vary by two orders of magnitude. It is also noted that the degree of uv or fl quenching increases with time and with decrease of Cu : CFCA ratio. These results are not surprising considering the influence pH, ionic strength and kinetic factors, on the complexation reaction between CFCA and Cu^{2+}. Earlier studies on the binding ability of CFCA are contradictory. Some investigators[41,42] reported that CFCA only forms simple monomeric complex with Cu(II) probably through catechol type coordination. The following K values for Cu-CFCA complexes were reported; $k_{101}= 7.08\times10^{12}$, $k_{102}= 5.5\times10^{22}$ and $k_{111}= 6.76\times10^{17}$. These experiments were conducted in 0.1 M Na_2ClO_4 at pH values ranging from 4.5 to 7.8. Other investigators[40,43–45] demonstrated that CFCA easily forms oligomeric structures with Cu^{2+} ions. Conditional stability constants ranging from $k_{101}= 5.01\times10^{12}$ to $k_{203}= 1\times10^{38}$ were reported. Our data suggests the formation of oligomeric and polymeric CFCA and Cu-CFCA complexes. Application of the DCI method to CFCA indicates that; i) under the experimental conditions used, a more hydrophobic Cu-CFCA complex is formed at molar ratio of 1:1 or higher; ii) the complex peak has its own characteristic t_R and uv absorption bands, however the charge transfer band characteristic of Cu(II)-catecholate interaction was not detected in the uv scans of the Cu-CFCA complex peak; iii) the stability of Cu-CFCA complex increases with time and with increase of CFCA ratio.

FA and FA-Cu(II) complexes

Time response chromatograms with FA or Cu-FA solutions at 24, 48, 96 and 144 hrs, did not show measurable changes. Figures 4-A shows chromatograms and uv-vis scans of free FA and Cu-FA (1:1) at pH 7. The free FA chromatogram shows partial resolution of seven regions of response (peaks). The Cu-FA chromatogram shows the resolution of the same number of peaks as the free FA but with reduced absorption intensities. The scans of Cu-FA peaks are indistinguishable from those of the free FA. The corresponding fl chromatogram is shown in Figure 4-B. The figure shows the resolution of the same peaks except, that peaks 2 and 3 are not resolved. The Cu-FA fluorescence chromatogram shows variable degrees of

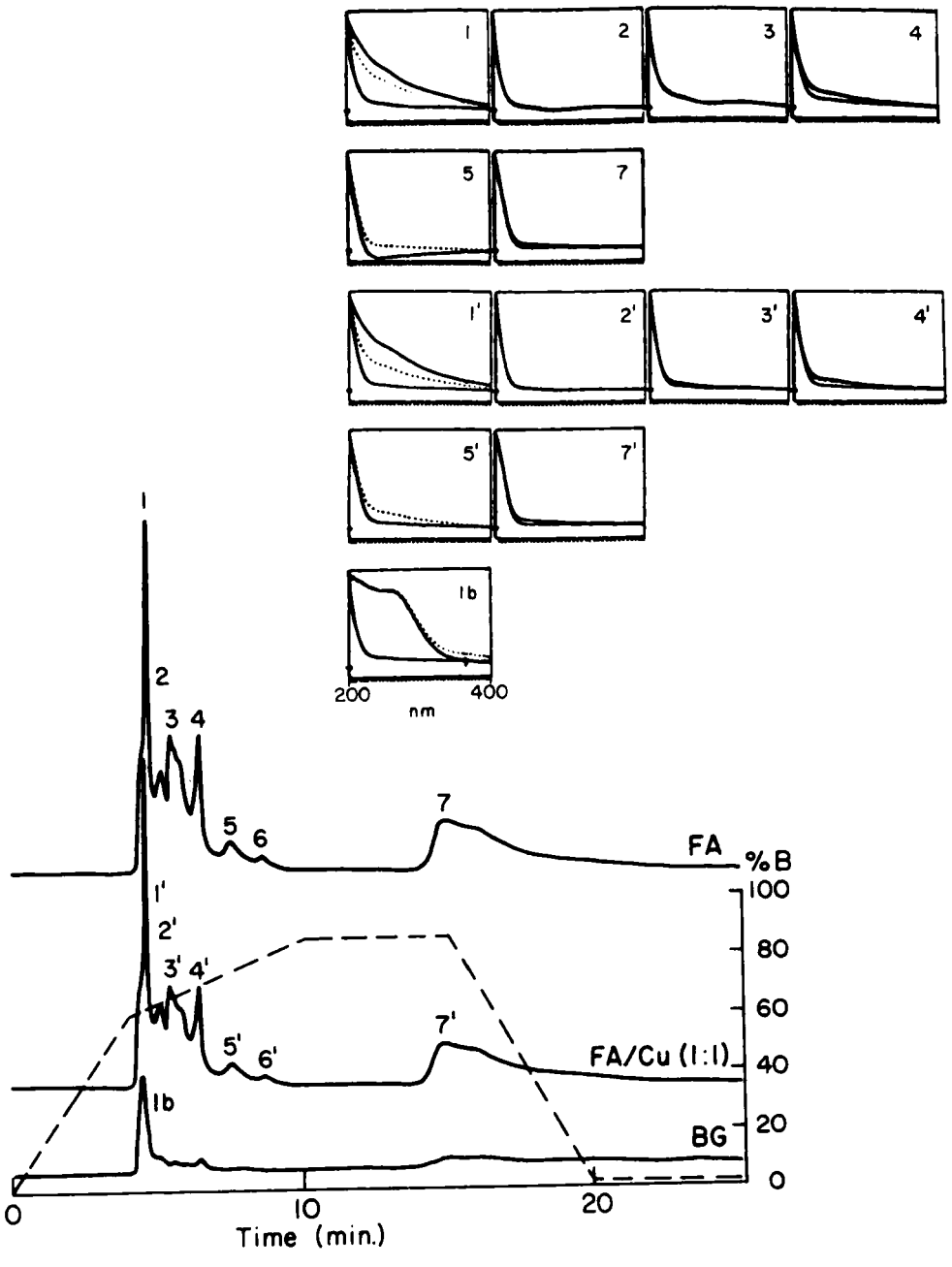

Figure 4-A Chromatogram (254 nm) and uv scans of 20 ul 2.22×10^{-4} M free and Cu-FA (1:1) in phosphate buffer. Experimental conditions are shown in Table 2, column Nova-Pak, gradient program III.

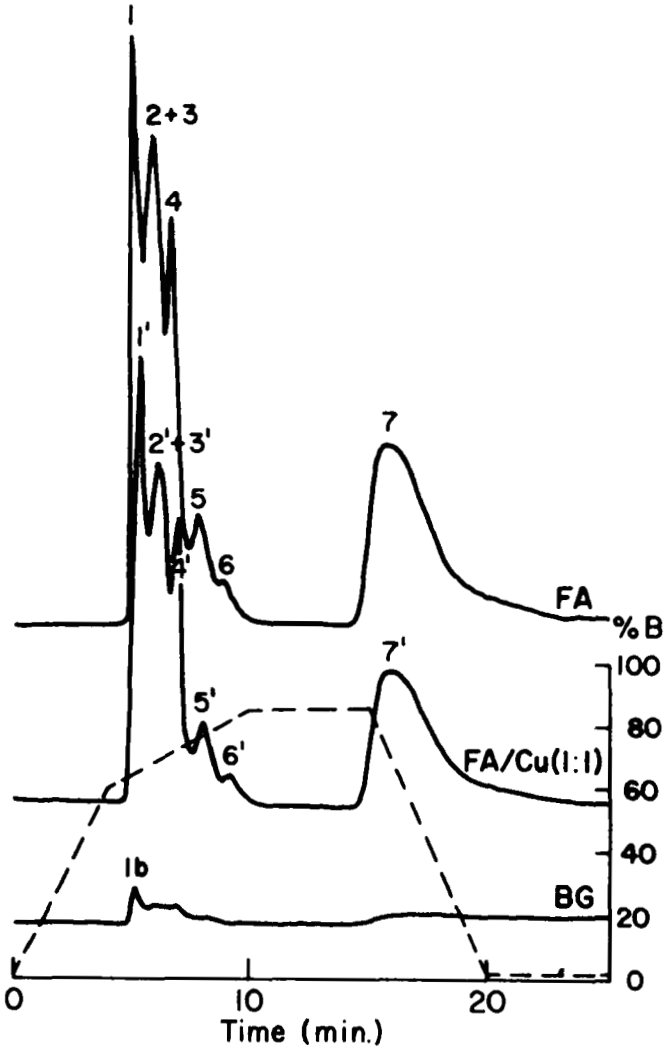

Figure 4-B Fluorescence chromatogram ($_{ex.}$ λ273, $_{em.}$ λ389 nm) of 20 ul 2.22×10^{-4} M FA and Cu-FA (1:1) in phosphate buffer. Experimental conditions are shown in Table 2, column Nova-Pak, gradient program III.

fl quenching. Results of the Cu-FA recovery experiment are shown in Table 4. The ionic Cu^{2+} was detected only in the background fraction and represented 17.90% of the Cu introduced. The fraction corresponding to hydrophilic peaks contains 41.0% of the total Cu, while the hydrophobic fraction contained 28.2% of the total Cu. The incomplete recovery of Cu could be due to the irreversible adsorption of Cu^{2+} on the stationary phase or due to the mechanical loss during the freeze drying steps. However a total recovery of 87.2% is considered satisfactory yield in preparative HPLC work[37].

Table 6 Conditional stability constants of Cu-FA fractions separated by RP-HPLC.

Peak No.	*HPLC-Fl*	*HPLC-UV*
1	x = 0.16 $K_1 = 1.14\times10^3$	x = 0.23 $K_1 = 1.78\times10^3$
2	x = 2.90 $K_1 = 2.38\times10^3$	x = 0.22 $K_1 = 1.76\times10^3$
3	—	x = 0.24 $K_1 = 1.82\times10^3$
4	x = 0.23 $K_1 = 1.69\times10^3$	x = 0.29 $K_1 = 2.39\times10^3$
5	x = 0.22 $K_1 = 1.68\times10^3$	x = .27 $K_1 = 2.12\times10^3$
6	x = 0.083 $K_1 = 5.28\times10^2$	x = 0.28 $K_1 = 2.25\times10^3$
7	x = 0.22 $K_1 = 1.65\times10^3$	x = 0.20 $K_1 = 1.45\times10^3$

Cu-FA solution (1:1), FA = Cu = 2.22×10^{-4} M in pH 7 buffer.
UV λ 254 nm, Fluo. λ_{ex}273 nm and λ_{em} 389 nm.
Chromatograms are illustrated in Figures 4-A and 4-B.

Efforts were made to use the areas under the chromatographic peaks in Figures 4-A and 4-B to calculate the conditional stability constants for each peak (site), using the method described earlier. The value of uv or fluorescence quenching (x) and the corresponding K are shown in Table 6. The method has several inherent sources of errors due to; i) assumption of uniform uv absorption or fl emission efficiencies for all peaks (binding sites); ii) incomplete resolution of the peaks; iii) possible shift of the equilibrium during the HPLC separation. Regardless of these limitations the data in Table 6 are reproducible and are in good agreement with K1 values calculated from total fl scanning or Cu analysis by AA. Complexation sites are likely to proceed from the more hydrophilic and acidic site to the hydrophobic and neutral sites. Earlier research on separation of free FA by RP-HPLC[36–39] has revealed that constituents of FA can be resolved into at least 4 hydrophilic acidic constituents which represented 40% of FA constituents, and several hydrophobic constituents which represented 30 percent the total FA. Furthermore RP-HPLC of a number of organic carboxylic, phenolic and keto acids with pk's from 1.9 to 10.29 were successfully separated on the same column[37]. For heterogenous polyfunctional ligands such as FA developing structure-retention information could lead to better understanding of metals complexation with natural ligands.

Several important conclusions can be derived from this experiment; i) FA can be separated into seven peaks (binding sites) by gradient RP-HPLC, ii) All the binding sites participate in complexation with Cu^{2+} as indicated by the quenching of the resolved peaks, iii) No major changes occur in the t_R of the peaks upon complexation indicating minor changes in the molecular volume or surface areas of the complexed molecules, iv) Conditional stability constants of FA compounds, calculated from the fl or uv spectra in the DCI

method, are comparable. The results of these experiments demonstrate an experimental framework to separate, and possibly identify the nature of binding sites in complex polyfunctional ligand such as FA.

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