SIMULTANEOUS DETERMINATION OF METALS USING PHASE-RESOLVED FLUORESCENCE SPECTROSCOPY

By

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DEDICATION

Dedicated to the Memory of

Margaret Raether

and to

Pamela Jean Vitense

(and new memories).

PREFACE

The work described in this dissertation applies the principles of phase-resolved fluorescence spectroscopy to the simultaneous determination of mixtures of metals after they were complexed with several different types of chelating agents. This particular application is one approach used to overcome the problem of spectral overlap that occurs when using fluorescence chelation as a detection technique for metal ion determination.

I would like to acknowledge the support of the Army Research Office for funding much of this work. I would also like to acknowledge the Chemistry Department of Oklahoma State University for support through teaching assistantships and scholarships throughout my graduate career.

I wish to thank several people without whose help this project would have been much more difficult. First, I would like to thank my thesis adviser, Dr. Linda McGown, whose guidance, support, motivation, prodding, and most importantly friendship brought me to the position that I am today. Also thanks go out to all the members of the lab group, who made work an

enjoyable, stimulating, and sometimes very strange experience.

I would also like to thank the other members of my committee, Drs. Horacio Mottola, Neil Purdie, and E. C. Nelson for their assistance when asked. I would like to particularly thank Dr. Mottola for his help whenever needed, even when that information required was in no way related to this work, and also for serving as an example of excellence in the classroom and laboratory.

The support of the faculty at Cameron University was appreciated. The gentle reminders to finish, and the genuine pleasure expressed when I finally did finish writing helped me feel that I had accomplished something.

Last, but certainly not least, I would like to thank my parents for their support throughout my long college career, one that at times they thought would never end. And of course, for everything, thanks pjv.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF SYMBOLS

ϕ	Phase delay
ϕ_D	Detector phase angle setting
τ_m	Lifetime calculated from demodulation data
τ_p	Lifetime calculated from phase delay data
ω	Angular modulation frequency

NOMENCLATURE

DPFH	Di-2-pyridyl ketone 2-furoylhydrazone
FAPH	2-furaldehyde 2-pyridylhydrazone
FLAPH	2-fluorenaldehyde 2-pyridylhydrazone
HCAQ	1-hydroxy 2-carboxyanthraquinone
INBH	1-isonicotinoyl-2-(2',4'-dihydroxy)-benzylidene hydrazine
LG	Lumogallion
NNLS	Non negative least squares
OBSH	N,N'-oxalylbis(salicylaldehyde) hydrazone
PABTH	Pyrocatechol-1-aldehyde 2-benzothiazoyl hydrazone
PAHP	Pyridene-2-carbaldehyde 2-pyridylhydrazone
PCAPH	Pyrocatechol-1-aldehyde 2-pyridylhydrazone
PCASH	Pyrocatechol-1-aldehyde salicyloyl-hydrazone
PRFI	Phase-resolved fluorescence intensity
PRFS	Phase-resolved fluorescence spectroscopy
QS	5-sulfo-8-hydroxyquinoline
SATCH	Salicylaldehyde thiocarbohydrazone
SATSC	Salicylaldehyde thiosemicarbazone
SoAP	Salicylidene-o-aminophenol
TMK	Trimethoxy kaempferol

CHAPTER I

INTRODUCTION

The use of chelating agents for metal ion determination using fluorescence measurements has been known since 1867 when Goppelsroeder used morin to qualitatively identify aluminum (1). Since that time thousands of analytical schemes have been developed for both qualitative and quantitative determinations of metals via chelation to form a fluorescent product.

Although a large number of metal ions will form chelates, and many chelators are known, only some of these may be used for fluorescence analysis. In general, the transition metals with partially filled d orbitals cannot be determined fluorimetrically (2-4), as these orbitals decrease (or eliminate) fluorescence by enhancing intersystem crossing. This allows more time for radiationless de-excitation modes to compete with photon-emitting modes for return of the excited molecules to the ground state.

Since the fluorescence of the complex in most cases, the lanthanides being an exception (5), is due to the electronic characteristics of the chelator, only chelators with certain structural features lend

themselves to fluorescence analysis. These features include either a high degree of conjugation of double bonds or an electron donor in the chelate, leading to $\pi - \pi^*$ or $n - \pi^*$ electronic transitions. The fluorescence from $n - \pi^*$ transitions is relatively weak, while the fluorescence from $\pi - \pi^*$ transitions is much stronger. The presence of a heteroatom in the ligand is also important for formation of the metal-chelate bond. When a bond is formed between the heteroatom and the metal ion, the characteristics of the excitation are often changed such that a weak $n - \pi^*$ excitation in the non complexed ligand becomes a much more intense $\pi - \pi^*$ transition in the metal chelate (2,5).

Many metal chelates do fit the above criteria. The metals of the IIIA family are the most prolific at forming fluorescent chelates. Of these metals, gallium and aluminum are the most likely to form fluorescent chelates. In a recent review, twenty-one percent of the determinations reported were for Group IIIA metals. Of these, fifty-three methods were reported for gallium determinations, and fifty-one methods were reported for aluminum determinations (2).

Substituents on the chelator that are not directly involved in the metal-ligand interaction may modify the binding characteristics of the ligand and the fluorescence properties of the complex (5). A strongly

electron-withdrawing group may reduce or eliminate fluorescence by increasing the π-π^* energy level difference. An electron donor likewise may increase the fluorescence by decreasing this energy difference.

Shifts in the excitation and emission spectra may also occur as the complexity of the ligand increases (5). As more complex groups are added to a ligand, a bathochromic shift is likely to occur. Since the magnitude of these shifts is dependent almost exclusively on the ligand, one of the problems that arises in these analytical methods is that of spectral overlap. If a chelator will combine with more than one metal ion there is typically a high degree of (if not complete) overlap of the resultant excitation and emission spectra. This is observed since the electronic characteristics, and therefore the observed fluorescence parameters, of the complex are mainly from the chelator (5). Therefore, various schemes have been developed to allow determination of mixtures of metal ions, including separation of the metals before or after chelation, determinations using derivative spectroscopic techniques (6), and determinations based on fluorescence lifetime differences between the different chelates (7).

The problem of spectral overlap is the focus of this dissertation. In this work, we demonstrate the use of phase resolved fluorescence spectroscopy (PRFS)

as a tool to accomplish the simultaneous multicomponent determination of metals. Using PRFS allows one to exploit a difference in fluorescence lifetimes, and it has been applied to the determination of up to six fluorescent constituents in a multicomponent mixture (8,9) and to the determination of phenobarbital in an immunoassay scheme (10). The theory and limitations of PRFS will be discussed in Chapter III.

Earlier work by Lytle et al. (11) has shown that, because of the heavy atom effect, a difference in the fluorescence lifetimes of metal chelates should occur. This dissertation confirms that there is indeed a fluorescence lifetime difference for the species from different metal chelates for several different chelator types. The categories examined are oxine derivatives, Schiff's base reagents, polyhydroxy flavones, and azo reagents. Further, the fluorescence lifetime differences are sufficient for all of the types of chelators examined to allow simultaneous determinations of two component systems of metals based solely upon this difference. These results are presented in Chapter IV.

CHAPTER II

A REVIEW OF FLUORIMETRIC METHODS FOR DETERMINATION OF METALS AFTER CHELATION

Equilibrium Methods of Determination

There are a large number of fluorimetric determinations of metal chelates in which the systems are allowed to reach a chemical equilibrium, and the fluorescence is then measured. In general these methods have the advantage of being relatively simple, both in procedure and fluorescence measurement. However, if the chelator forms a fluorescent product with more than one species it may be impossible to distinguish the individual contributions. The inability to distinguish fluorescence from different metals is one of the main problems with the equilibrium techniques.

In spite of the problems involved with equilibrium determinations, there are new equilibrium methods constantly being developed. Syntheses of chelators which are more selective for one metal in the presence of interfering metals and chelators which have higher

quantum yields are only two of the areas in which new methods are being developed.

Fernandez-Guiterrez and Munoz de la Pena (2) recently reviewed several methods for the determination of metals via fluorescence chelate formation, and much work has been done in this area since the publication of their review. The emphasis of this section will be on recent developments for equilibrium fluorimetric determinations. Table 1 lists the chelators that will be discussed in this section, and includes the reported limits of detection and linear ranges for the determinations.

Determinations of Aluminum

Aluminum has been found to form fluorescent metal chelates with a vareity of ligands (4,5), and a large number of equilibrium fluorimetric methods have been developed for the determination of aluminum (12-31). A description of a few of these methods follow.

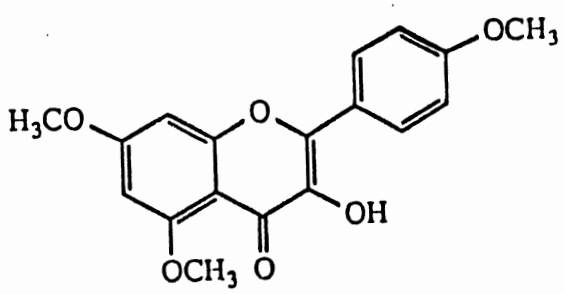
Zel'tser et al. (11) propose the use of trimethoxykaempferol (TMK), a naturally-occurring polyhydroxy flavone isolated from plant raw material, for the determination of aluminum. The structure of TMK is shown in Figure 1. Determinations of trace amounts of aluminum in tin and in natural waters using TMK as a chelator are shown. The structure of TMK is very similar to that of morin, for which a large number

TABLE 1

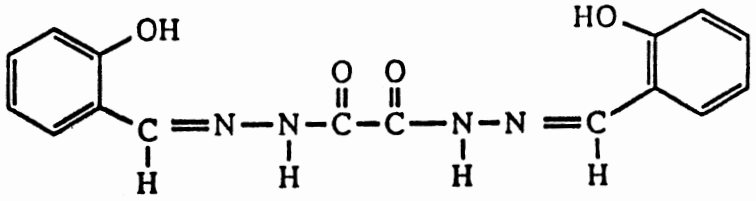
LIMIT OF DETECTION AND LINEAR RANGE FOR THE CHELATORS DISCUSSED

Chelator	LOD (ng/ml)	Linear Range (ng/ml)
For Aluminum[b]		
DPFH	4	10 - 100
HCAQ	2	3 - 250
INBH	0.5	0 - 5000
Pamonic acid	19	80 - 1700
OBSH	(a)	0 - 200
TMK	0.14	(a)
For Gallium[c]		
HCAQ	(a)	50 - 500
PABTH	0.24	1 - 80
PAHP	(a)	10 - 500
SATCH	2	3 - 30
SATSC	3	10 - 10
For Zinc[d]		
FAPH	(e)	(e)
PCAPH	5	12 - 250
PCASH	1.5	10 - 800
SATCH	10	15 - 1300

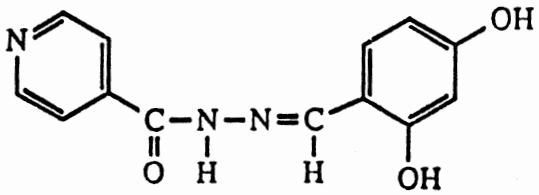
[a]Not reported.
[b]One ng/ml aluminum = 37 nM.
[c]One ng/ml gallium = 14 nM.
[d]One ng/ml zinc = 15 nM.
[e]See Table 9.



TMK



OBSH



INBH

Figure 1. Some chelators used for aluminum determinations.

of aluminum determinations have been described (15-23), and which is one of the chelators used in the studies described in this dissertation.

The reported detection limit of 0.14 ng/ml is at least three times lower than that of any other chelator discussed in this section. TMK also has a higher tolerance for many of the metals which interfere with the determination of aluminum using other structurally similar chelators (14).

The intensity of the fluorescence of native TMK at the wavelengths used for the determination was not reported, although there is mention of a bathochromic shift for the complex, indicating that there may be background fluorescence. Also, the authors mention that maximum fluorescence was obtained when the solutions were first mixed together. This indicates that there is some decrease in the fluorescence over time; however, the severity of this decrease is not mentioned.

There are also several interfering species. It is reported that many of them can be masked, although no determinations were shown in which masking was done. The final composition of the solvent for the system is only 10% water. This raises the effective limit of detection and minimum concentration of aluminum which can be determined (assuming that the aluminum is dissolved in water) by at least a factor of 10.

Ariza et al. (24) have studied N,N'-oxalyl-bis(salicylaldehyde hydrazone) (OBSH) as a fluorimetric reagent for aluminum determinations in naturally-occurring mineral waters. The structure of OBSH is also shown in Figure 1. The reported linear range for these determinations is 0-200 ng/ml in a 60% DMF, 40% water solvent. No limit of determination is reported, but a 'sensitivity' of 5 ng/ml is given (Table 2). It is more likely that a table of limits of detection is given, since the units reported are not valid units for sensitivity.

There was no shift in the fluorescence of OBSH upon complexation with aluminum or any of the interfering species. These complexes exhibited a constant fluorescence for several hours at acid and neutral pHs after heating. The pH chosen for the fluorimetric determinations was 5.0, since at this pH there was very little change in intensity with change in pH, and it was also within the pH range that the fluorescence was at a maximum.

Several compounds were found to interfere, with gallium and indium having the highest positive interferences reported (less than 20 ng/ml for a 20 ng/ml sample of aluminum). This interference is due to the fluorescence metal chelates which are formed with both metals. The intensities of Ga-OBSH and In-OBSH

TABLE 2

DETECTION LIMITS OF COMMON REAGENTS FOR THE FLUORIMETRIC DETERMINATION OF ALUMINUM

Reagent	Detection Limit (ng/ml)
Salicylidene-o-aminophenol	0.3
Mordant Blue 9	0.5
Flazo Orange	1
N-Salicylidene-2-amino-3-hydroxyfluorene	1
OBSH	5
Alizarin Garnet R acid	7
3-Hydroxy-2-napthoic acid	10
Lumogallion	14
Pentachrome BBR	20
Pentachrome VSW	20
Morin	50
Quercetin	50
8-Hydroxyquinoline	100
Cumarin derivatives	200

All values from Reference 24.

were similar, and about 40% that of the intensity of the Al-OBSH complex.

Some of the traditional masking agents for aluminum determinations, such as tartrate and EDTA, interfere with the Al-OBSH system and must be avoided. In addition to those species which gave a positive interference, interferences from 3-fold excess of Pd(II), Th(IV), and Bi(III), and 5-fold excess of V(V), Cu(II), Fe(III), and Ti(IV) were also reported.

The procedure was both simple and straightforward. However, as mentioned earlier, there was no limit of detection reported for the system. Also, since the final solvent was only 40% water, the effective limits for the linear range would increase, as would the limit of detection.

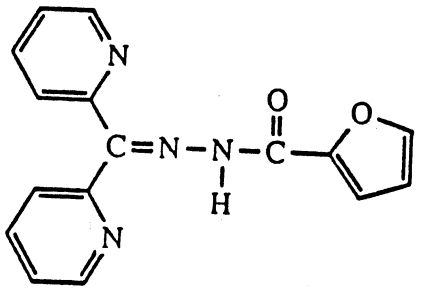
1-Isonicotinoyl-2-(2',4'-dihydroxy)benzylidene hydrazine (INBH, Figure 1), a reagent which is somewhat similar to OBSH, was used by Vasilikiotis et al. for the fluorimetric determination of aluminum (25). The samples were analyzed in a water-ethanol mixture with the pH of the aqueous phase adjusted to 5.0 before addition of the ethanol. The reported LOD is 0.5 ng/ml.

Formation of the aluminum-INBH complex exhibited first-order kinetics, with a rate coefficient of approximately 0.35 min^{-1} for the complex formation reaction. The rate of formation can be increased by

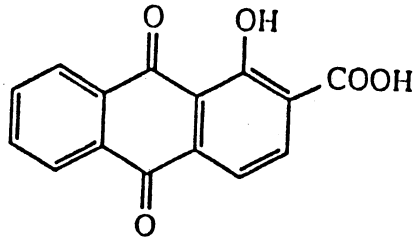
heating the mixture to 60 ^{o}C, giving complete formation of the complex in 15 minutes. The samples must subsequently be cooled to increase the quantum yield of the product.

This system does have a low limit of detection (0.5 ng/ml) and a large linear range for the determination (0 - 5000 ng/ml) relative to the other chelators (Table 1). The fairly rapid chelate formation at higher temperatures is also advantageous. The solution for the determination is 50% ethanol. This increases the effective minimum concentration of aluminum that can be determined in an aqueous sample. Three different natural water samples were analyzed. However, there was no independently determined value of aluminum for comparison, and there were no reported determinations of synthetic samples or samples containing known concentrations of aluminum.

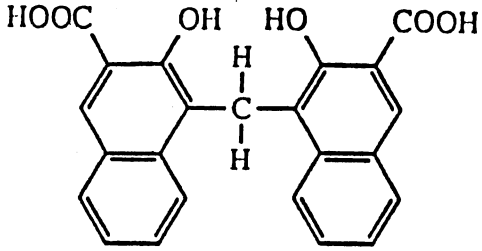
Another reagent for the determination of aluminum is di-2-pyridyl ketone 2-furoylhydrazone (DPFH, Figure 2). Determinations of aluminum with DPFH have been reported by Ordonez et al. (26), and were accomplished using both synthetic and natural samples of seawater. Synthesis of the reagent appeared to be quite simple. According to the authors, this is in contrast to the synthesis of a similar reagent, 3-hydroxy-pyridene-2-aldehyde 2-pyridylhydrazone (27).



DPFH



HCAQ



Pamonic Acid

Figure 2. More chelators used for aluminum determinations.

The detection limit for this method is 4 ng/ml. This would appear to be quite high, but since the final solvent composition is 96% water this value is a better indication of the actual detection limit for a sample. However, the linear range is from 10 to 100 ng/ml, which is fairly small, especially relative to some of the other proposed methods (Table 1). The plot of intensity vs. amount of ligand (Figure 3) never does exhibit a plateau. This may be why the linear range is small.

A method for the determination of aluminum in real samples of Portland cement and aluminum bronze was reported by Salinas et al. (28). The reagent for these determinations is 1-hydroxy-2-carboxyanthraquinone (HCAQ, Figure 2). The method works well for the analyses proposed, as shown in Table 3. In both cases there was a positive error. This could be due to the natural fluorescence of HCAQ or the presence of other metal species which form fluorescent products.

There is a very narrow concentration range of chelator (30 to 60 μM) which can be used for these determinations. There is also significant fluorescence from the non-complexed HCAQ, and the complex is stable for only about 6 hours. The final composition of the mixture for the determination is 40% water (in an ethanol/water mixture), again increasing the effective limit of detection and linear range for an aqueous

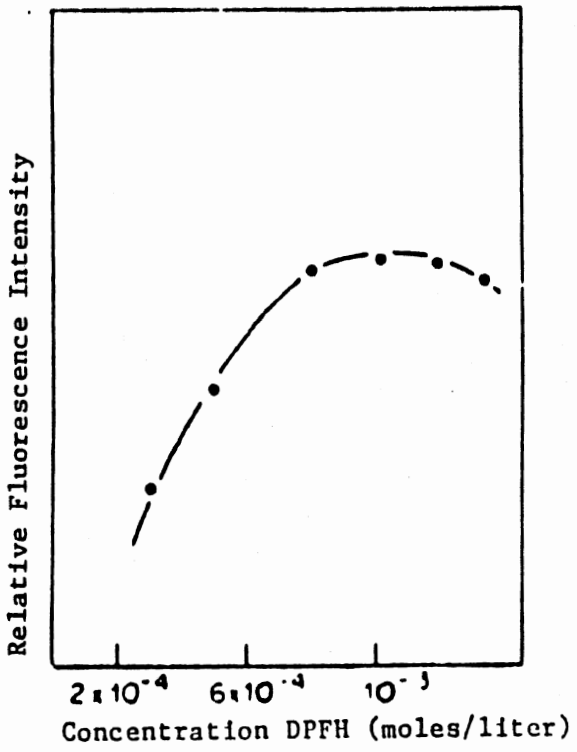


Figure 3. Intensity vs. ligand concentration for Al with DPFH

TABLE 3

DETERMINATION OF ALUMINUM IN SAMPLES USING HCAQ

Sample	Actual % Al(III)	Determined % Al(III)	Standard Deviation[a]
Portland Cement	5.35	5.50	0.08
Aluminum Bronze	8.80	8.93	0.09

[a]Six determinations per sample.

All values from Reference 28.

sample. To utilize the entire reported linear range, two different concentrations of chelator must be used, one from 3 to 25 ng/ml and another from 25 to 250 ng/ml.

Pamonic acid (2,2'-dihydroxy-3,3'-dicarboxy-1,1'-dinapthylmethane, Figure 2) has been proposed for use as an analytical reagent for the fluorimetric determination of aluminum by Casassas et al. (29). This molecule has the same functional group as 3-hydroxy-2-napthoic acid, however, determinations with the latter reportedly have a low tolerance ratio for iron (30). The authors claim that this method is free from interferences from iron.

The pamonic acid method appears to be the least important of the methods discussed for aluminum. The detection limit is quite high (19 ng/ml), and there is some question as to the actual quantitation limit. On the first page of the article the value reported is three orders of magnitude less than the value reported in the body of the article. The larger value is consistent with the other reported values (limit of detection and linear range), and is probably the actual value. The linear range for the determination is fairly large (80 to 1700 ng/ml), but not as large as that for INBH. The final working solution is about 70% dioxane, and again the reported detection limit and linear range is for the final solutions, not the actual

concentration of aluminum in the sample that one would be able to determine.

The intensity of the fluorescence for the chelate is pH dependent, and there was no buffer used for the system. It was reported that acetate buffer quenched the fluorescence, and that other buffers caused precipitation of the complex. It would appear from the plot of intensity vs. pH (Figure 4) that any small variation in pH would cause a dramatic change in the intensity.

Gallium and beryllium interfere at very low concentrations, and the method is not free from interference from iron as was stated by the authors; the reported tolerance ratio for iron (Fe/Al) is 4.5 (30). A complete list of the reported interfering species is given in Table 4. As was the case with HCAQ, two concentrations of chelator were used to obtain the reported linear range.

Determination of Gallium

Gallium also forms fluorescent chelates with many chelators. In fact, many of the reagents that are used for aluminum determinations can also be used for gallium determinations (2). Unfortunately, this means that even a small amount of aluminum present in a sample may interfere with the determination of gallium.

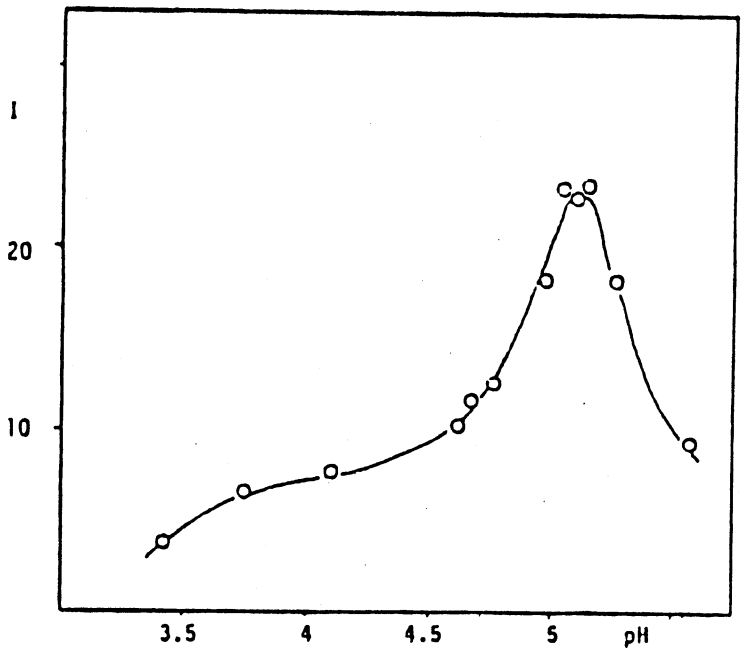


Figure 4. Intensity vs. pH of the aluminum-pamonic acid chelate.

TABLE 4

INTERFERING SPECIES FOR THE DETERMINATION OF ALUMINUM WITH PAMONIC ACID

Ion	Tolerance limit[a]	Tolerance ratio[b]
Ga(II)	<0.1	<0.07
Be(II)	<0.1	<0.5
Hg(II)	0.2	0.05
Mg(II)	0.2	0.4
Mn(II)	0.25	0.23
In(III)	1.0	0.45
Zn(II)	1.0	0.8
Ca(II)	1.0	1.25
Cu(II)	2.5	2
Fe(III)	5	4.5
Pb(II)	13	3
NO_3^-	>500	>403
NH_4^+	>500	>1400
Cl^-	>3550	>5000
K(I)	>3900	>5000

[a] Units are mg/l.
[b] [interfering species] / [Al]

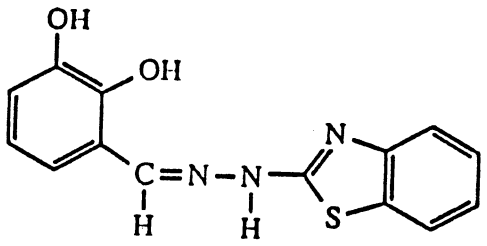
All values from reference 29.

The first reagent for the determination of gallium to be discussed is HCAQ (31). This is the same reagent discussed earlier for the determination of aluminum. Salinas et al. have been able to successfully determine trace amounts of gallium using HCAQ (28). The limit of detection for the method is not reported. However, the linear range for the determination is reported to be 50 to 500 ng/ml. Since the complex was temperature sensitive, the temperature of the system was thermostatically controlled.

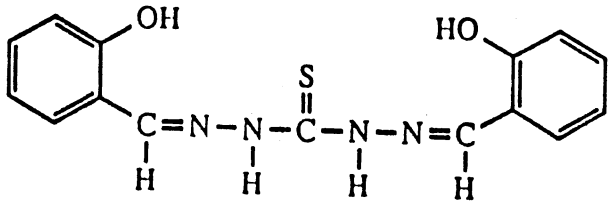
There were several interferences for the method, including acetate and phosphate buffers which completely quench the fluorescence of the product. For a 200 ng/ml gallium sample, seventeen cations interfered at the μg/ml level or below, and of these twelve interfered at a level reported as less than 200 ng/ml. No testing of interfering species at concentrations of less than 200 ng/ml was reported.

The linear range is reported as 50 to 500 ng/ml, but this is in a 70% ethanol solution. The actual concentration range of the metals able to be determined would be higher than the 50 to 500 ng/ml reported. There is also a significant blank contribution to the signal. A determination of only one concentration of gallium (300 ng/ml) was reported.

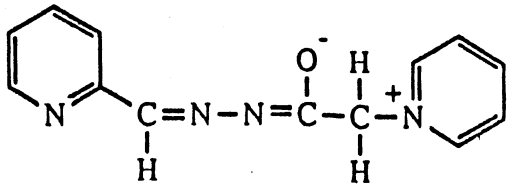
Pyrocatechol-1-aldehyde 2-benzothiazoylhydrazone (PABTH, Figure 5) was reported by Afonso et al. (32) as



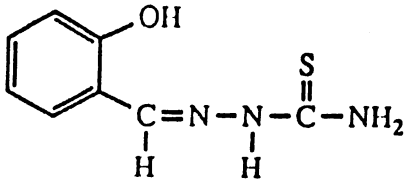
PABTH



SATCH



PAHP



SATSC

Figure 5. Some chelators used for gallium determinations.

a reagent for the determination of gallium in blood and urine. The determinations were carried out in a water:DMSO mixture. The limit of detection for the procedure was 0.24 ng/ml, and the reported linear range was 1 to 80 ng/ml. The method was successfully applied to the determination of gallium in the above matrices, as shown in Table 5. The determination in these body fluids is important because gallium is used as a tumor scanning agent and exhibits antitumor activity. Since it is toxic, there is a need for analyses which can be carried out simply and accurately in body fluids.

All values from Table 5 are from recovery studies, which show the ability to determine gallium in these particular matrices, since what was actually determined was the amount of "spiked" gallium in these fluids. The authors state at the end of the paper that "... the proposed method would be effective for the spectrofluorimetric determination of gallium in these kinds of samples." (32). Therapeutic concentrations of gallium in human sera can range from 100 to 3000 ng/ml (33). Toxicity studies in rats show that higher concentrations of gallium in rats were potentially fatal (34).

To obtain the reported linear range for the determinations it was necessary to use two different concentrations of the chelator. The determinations were done in a 50% water, 50% DMSO mixture. Again, to

TABLE 5

SPECTROFLUORIMETRIC DETERMINATION OF GALLIUM IN HUMAN URINE AND BLOOD SERUM SAMPLES WITH PABTH

Ga added[a]	Ga found[a]	Recovery %
0	0	----
1.0	0.9	90.0
3.0	2.9	96.7
6.0	6.0	100.0
9.0	9.3	103.3
12.0	11.7	97.5
15.0	15.1	100.7

[a]Concentrations are in ng/ml.

All values from Reference 32.

get a more accurate picture of the actual linear range and limit of detection one must increase the reported values by about two.

There were also many interfering species in the determinations with this reagent. Twenty eight interfering species were reported at the 10 ng/ml level for a 52 ng/ml sample. The strongest interfering species were zinc (2.5 ng/ml) and aluminum (less than 2.5 ng/ml). Also, the intensity of the chelate was constant for only one hour.

Urena et al. have also developed a method for the determination of gallium in biological samples (35). The method uses salicylaldehyde thiocarbohydrazone (SATCH, Figure 5) as the chelating agent. Recovery studies were performed, demonstrating the ability to determine gallium in samples of bovine liver, kidney, and brain, and human urine.

The pH dependence of the chelate was studied, and it was found to exhibit fluorescence at both high and low pHs. The studies were done at a pH of 2.3, since at this pH, unlike the high pH range, the reagent showed no appreciable fluorescence (Figure 6). The fluorescence intensity reached a maximum after 25 min., and remained constant for at least 4 hs. The maximum fluorescence for the product in the working solvent, which was 52% ethanol and 48% water, occurred when a 558-fold excess of chelator was used. Under these

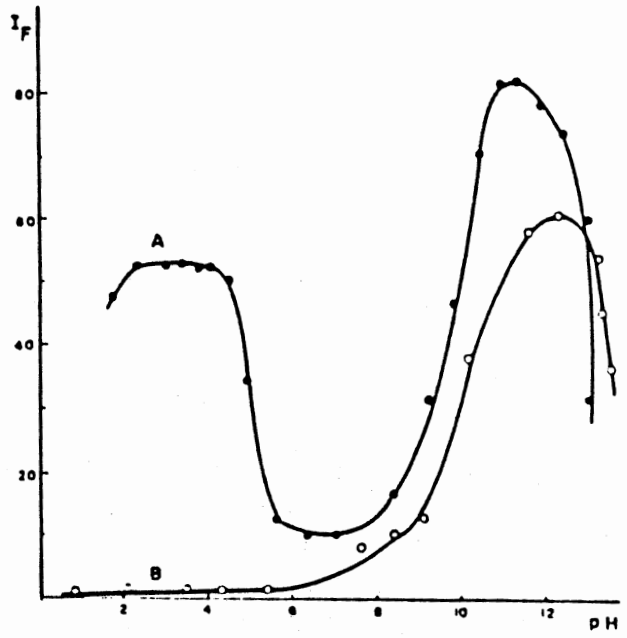


Figure 6. Intensity vs. pH for SATCH (B) and for the gallium-SATCH chelate (A).

conditions the detection limit is 2 ng/ml, and the linear range for the determination is from 3 to 30 ng/ml.

Several species were found to interfere with the determination of a 15 ng/ml sample of gallium, as shown in Table 6. The chelator shows a good selectivity for gallium over aluminum (3000-fold excess) and over indium (100-fold excess). It was also found that the interferences from Cu(II), Fe(II), Fe(III), and Mo(VI) could be masked by suitable reagents to increase the amount of each tolerated, as also shown in Table 6.

A method has been reported by Mochon et al. (36) for the determination of gallium in aluminum, using pyridene-2-carbaldehyde Girard-P (PAHP, Figure 5). The method was used successfully for the determination of gallium with no other metals present and for the determination of gallium in a sample of aluminum. All solutions were in distilled - deionized water.

The detection limit for the method was not reported. There were two linear ranges reported: from 10 to 100 ng/ml and from 50 to 500 ng/ml, corresponding to two concentrations of chelator used for the determinations. The linear ranges are probably a fairly accurate indication of the actual concentrations of gallium which can be determined since the solutions are all aqueous, and the size of an aliquot for the determinations is not limited by the composition of the

TABLE 6

INTERFERING SPECIES FOR THE DETERMINATION OF GALLIUM WITH SATCH

Molar excess[a]	Species which interfere
5000	Na(I), K(I), Ba(II), Sr(II), Mg(II), Mn(II), nitrate, chloride.
3000	Li(I), Al(III), thiosulfate.
1000	Be(II), Cr(III), Ag(I), Zn(II), Cd(II), Th(IV), Pb(II), Tl(I), Zr(IV), Bi(III), Y(III), acetate, chlorate, sulfate, iodide, carbonate, sulfide, sulfite.
500	Ni(II), periodate, persulfate.
100	W(VI), Sc(III), In (III), arsenate, iodate, thiocyanate, nitrite.
50	Hg(II), Sn(II), Au(III), Tl(III), As(III), Sb(III), cyanide, permanganate.
10	Ce(IV), Fe(III), La(III), fluoride.
5	Cu(II), Fe(II), Mo(VI), V(V).

	Interferences which can be masked.			
Ion	no masking[b]	masking[b]	reagent	conc.[c]
Cu(II)	75	750	thiosulfate	15
Fe(II)	75	150	permanganate	2.25
			fluoride	0.15
Fe(III)	150	750	fluoride	0.15
Mo(VI)	75	150	fluoride	0.75

[a] [interfering species]/[gallium]
[b] Concentrations in ng/ml.
[c] Concentrations in μg/ml.

All values from Reference 35.

solvent. The system also appears to be more tolerant of low concentrations of interfering species than many other methods. Fe(II), Cu(II), and Sn(II), which create the highest interference to the determinations, all interfere at a level equal (in ng/ml) to that of the gallium ion.

The pH range for the fluorescence intensity maximum is very narrow, with the optimum pH being about 3.7. Variations of as little as 0.3 pH units can cause dramatic changes in the intensity, as seen in Figure 7. The complex is also very temperature sensitive. Earlier in the paper it is reported that a three-fold molar excess of aluminum can be tolerated, but according to the data presented in the paper an eight-fold molar excess can be tolerated.

The final gallium chelator to be discussed is salicylaldehyde thiosemicarbazone (SATSC, Figure 5), proposed by Stolyarov et al. (37). The thiosemicarbazone is much more selective for gallium than for aluminum because of the replacement of the carbonyl oxygen with a sulfur in the semicarbazone functionality. The pH range for the complexation is from pH 2 to pH 6 (38,39). However, the optimum pH for a particular determination is dependent on solution composition, especially the aluminum concentration, and is usually from 3.7 to 4.2. Determinations of gallium were done in several real systems, including

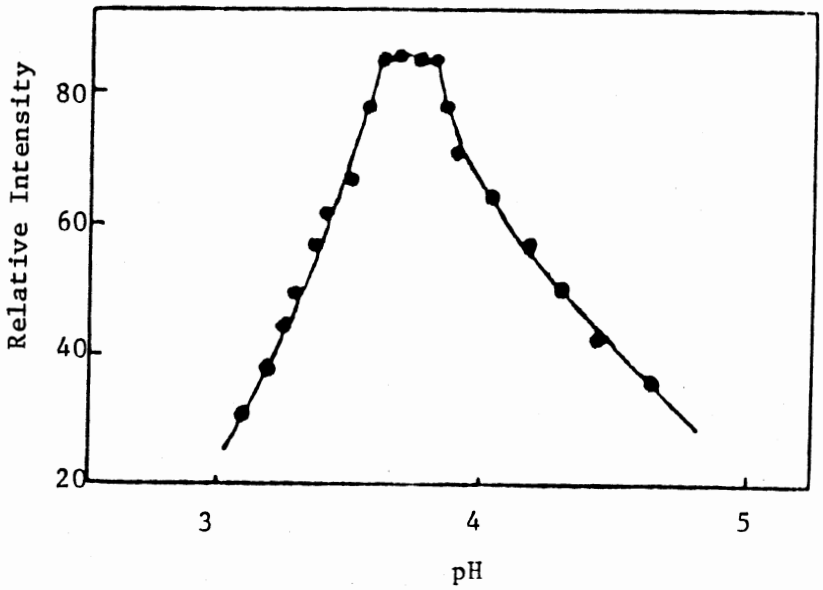


Figure 7. Intensity vs. pH for the Gallium-PAHP Chelate.

magnesium-based alloys, semiconductor alloys, and standard samples of silicate rock. The values for the determinations in various magnesium alloys are shown in Table 7.

The above method allows determination of gallium in samples with a large excess (100-fold to 1000-fold) of aluminum. This is one of the better tolerances of aluminum for the gallium chelators discussed. The detection limit of gallium using SASTC is 3 ng/ml (but up to 4 to 5 ng/ml in the presence of aluminum), which is slightly higher than the detection limit for the oxygen-containing analog (38,39). However, the oxygen analog is more selective for aluminum than gallium, so this increase in the detection limit is insignificant relative to the increased selectivity for the gallium. The linear range for the determination is 10 to 100 ng/ml in the presence of 1 to 10 mM aluminum.

As the concentration of aluminum in the sample increases, the detection limit of gallium also increases. In the proposed method it is necessary to match the concentration of the aluminum ion with that of fluoride in a 1:1 or a 1:2 ratio. This implies that the concentration of the aluminum in the sample is known, or at least can be approximated. The intensity of the complex is affected by so many factors that a calibration curve cannot be used. The only recommended

TABLE 7

DETERMINATION OF GALLIUM WITH SATSC
IN MAGNESIUM ALLOYS

Alloy	% Ga (SATSC)[a]	% Ga (other)[b]
1	0.34 ± 0.02	0.35 ± 0.02
2	0.60 ± 0.03	0.61 ± 0.02
3	0.49 ± 0.02	0.52 ± 0.03

[a]Eleven replicates per sample.
[b]Gallium by complexiметric determination.

All values from Reference 37.

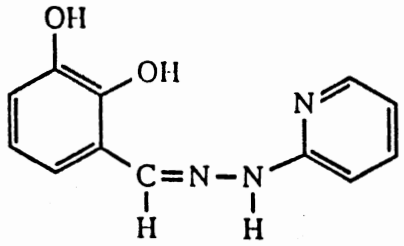
method for the determinations using SATSC is that of standard additions.

The only direct determination of gallium without prior separation presented is that of gallium in magnesium alloys. For the determinations in the other media the species in the solution containing the gallium are chromatographically separated. The final solvent for determinations is 50% aqueous ethanol. This would raise the effective limit of detection and linear range by at least a factor of two for the determinations.

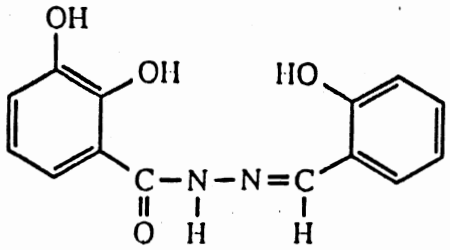
Determination of Zinc

Zinc is one of the transition metals that can be determined fluorimetrically after chelation, since the ionic form of zinc does not have a partially filled d electron orbital. This allows formation of complexes in which fluorescence, and not intersystem crossing, is the main pathway for the complex to exit an excited singlet state (2,5).

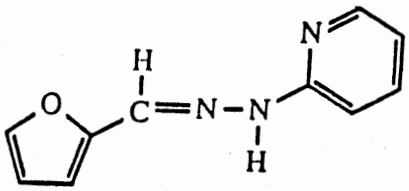
Afonso et al. (40) have developed a method for the spectrofluorimetric determination of zinc with pyrocatechol-1-aldehyde 2-pyridylhydrazone (PCAPH, Figure 8). Concentrations of zinc are determined from calibration curves. The intensity of the fluorescence was not sensitive to the ionic strength of the solvent in the concentration range examined.



PCAPH



PCASH



FAPH

Figure 8. Chelators used for the determination of zinc.

The uncomplexed chelator does fluoresce; however, the excitation and emission spectra of the chelator do not overlap significantly with the same spectra of the metal chelate, when both are in acidic media. A 50% aqueous ethanol solvent was chosen as a compromise between fluorescence intensity, which increases as the ratio of ethanol increases, and aqueous sample size for the determination.

Results for the determination of zinc in potable tap water from several cities in the Canary Islands were shown. The samples were also analyzed by atomic absorbtion spectroscopy for comparison purposes. Results for the analyses are shown in Table 8. The results from the independent methods were similar, and all values obtained were less than the maximum concentration of zinc allowed in potable water by the Spanish Food Directorate (1.5 ppm).

The detection limit for the method is 5.5 ng/ml, and the linear range is 12 to 250 ng/ml. To achieve the linear range, two separate concentrations of chelator were used. One concentration was used for determinations from 12 to 80 ng/ml of zinc, and the second for determinations from 80 to 250 ng/ml of zinc. Many species interfered with the determinations: twenty-five species interfered at or below an equal molar concentration of zinc.

TABLE 8

ANALYSES OF POTABLE WATER FOR ZINC

Sample	PCAPH[a]	AAS[a,b]
1	104	104
2	277	277
3	1295	1294
4	147	147
5	160	160
6	271	273

[a]All concentrations are ng/ml.
[b]Values from atomic absorbtion spectroscopy.

All values from Reference 40.

Afonso et al. (41) have also developed a method for zinc determination using pyrocatechol-1-aldehyde salicyloylhydrazone (PCASH, Figure 8). This method was used to determine zinc in alloys and in lubricating fluids. The method is sensitive to the presence of many species, however, interferences from copper and aluminum can be masked with suitable reagents. The masking was effective enough to allow the determination of zinc in an aluminum alloy without prior separation of the components, with zinc present in as low as 0.13% of the total composition of the alloy. The concentration of zinc in the samples was determined from a calibration curve.

The final composition of the solvent for the determinations was 60% ethanol. This was chosen as a compromise between fluorescence intensity, which increased as the percentage of ethanol in the solvent increased, and aqueous sample size. The detection limit for the method is 1.5 ng/ml, and the linear range is 10 to 800 ng/ml with two different concentrations of chelator. Twenty species interfere at or below an equal concentration of zinc, including Zn(II), Al(III), In(III), Cd(II), and Mg(II) with which PCASH forms fluorescent chelates.

The method worked very well for the four determinations reported, two in zinc alloys and two in lubricating oils. The results for the determinations

in the lubricating oils were compared to values from AAS, and the alloys were accompanied by a certified analysis in which the amount of zinc present was reported.

Cano Pavon et al. (42) have determined zinc in traces of biological materials, wine, and alloys with salicylaldehyde thiocarbohydrazone (SATCH, Figure 5). This is the same reagent that was used for the determination of gallium discussed earlier. The limit of detection was 10 ng/ml, and the working linear range for the determinations was from 15 to 1300 ng/ml, and is for a single concentration of SATCH. Many species interfere with the determinations, all at a much higher concentration of zinc (in ng/ml) than the level of zinc being determined. Also, the more common interferences, including Fe(II), Ga(III), Cu(II), Ni(II), Co(II), Hg(II), and Ag(I), can be masked.

The determinations in all matrices studied were for native concentrations of zinc, and not recovery studies. For the determinations in biological samples, the wet oxidation method by Bajo (43) was used, and compared with results from AAS. A possible problem with the method other than the presence of interfering species is the narrow pH range in which the fluorescence is at a maximum (Figure 9).

Zinc was determined by Garcia Sanchez and Hernandez Lopez (44) using 2-furaldehyde 2-pyridyl-

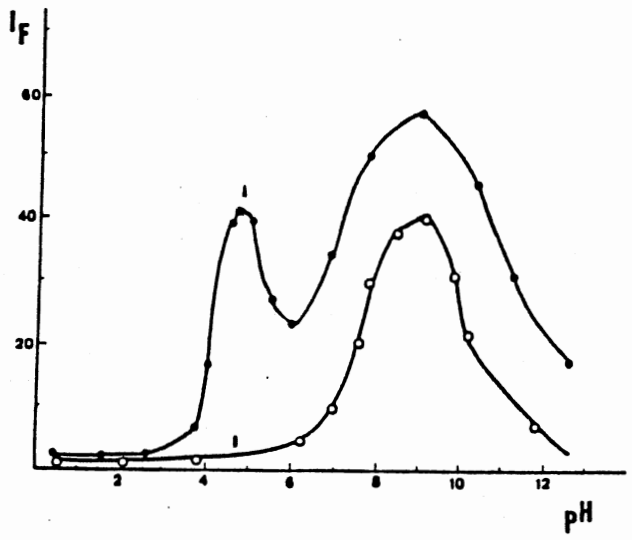


Figure 9. Intensity vs. pH for SATCH (B) and for the zinc-SATCH chelate (A).

hydrazone (FAPH, Figure 8). This study is noteworthy in that the authors compare the use of synchronous derivative fluorimetry to conventional (fixed excitation and emission wavelength) fluorimetric determinations. In synchronous scanning fluorimetry the spectrum is obtained by changing both the excitation and emission wavelengths, usually while maintaining a constant wavelength difference ($\Delta\lambda$). For these determinations the derivatives (both 1^{st} and 2^{nd}) of the synchronous spectra were obtained.

The final composition of the solution used for the determination was 30% ethanol. The concentration of ethanol was critical, since if there is less than 10% ethanol the solutions were turbid, and above 40% ethanol the fluorescence intensity decreased as the percentage of ethanol increased. The detection limits and linear ranges for the three methods are given in Table 9. Since the compound is temperature sensitive, the temperature was maintained at $15.0^{o}C \pm 0.5^{o}C$. The complex was also light sensitive, so all samples were stored in the dark once the chelator was added. The pH for the determinations was 12.5, and was maintained with a glycine buffer.

The technique was successfully applied to determinations of zinc in air samples and to recovery studies of zinc in porcine liver samples. The synchronous scanning approach for determinations was

TABLE 9

LIMITS OF DETECTION AND DETERMINATION AND LINEAR RANGE FOR THE NORMAL, FIRST AND SECOND DERIVATIVE DETERMINATIONS

Derivative	LOD[a]	LOQ[a]	LDR[a]
0^{th}	15	50	50-700
1^{st}	3	10	10-120
2^{nd}	5	15	15-120

[a]Concentrations are ng/ml.

All values from Reference 42.

compared to traditional masking agents in conventional spectrofluorimetry. For these determinations there was no significant improvement using the synchronous scanning techniques and it was found that the addition of masking agents is generally more efficient.

Determinations of other Metals

Many methods have been developed for the determination of metals other than those discussed above. All of the determinations are similar to those discussed in this chapter, and all have advantages and disadvantages associated with them. Recently developed methods include determinations of lithium (45), beryllium (17,46-48), zirconium (49), hafnium (49), niobium (22,50), tantalum (50), osmium (51), cadmium (52), and lead (53). These do not include methods which have been developed for the lanthanides, which can exhibit native fluorescence due to the transitions involving the f electrons of the metal, as well as fluorescence after chelation (2-4).

Application of Synchronous Derivative Spectroscopy to Multicomponent Determinations

A method that has been applied to the simultaneous determination of two metals after chelation is that of

synchronous derivative spectroscopy. This is the same technique examined for the determination of zinc with FAPH. It has been applied to the simultaneous determination of aluminum and beryllium mixtures using morin as the chelator (54), and also to the determination of gallium and zinc mixtures using SATCH as the chelator (6).

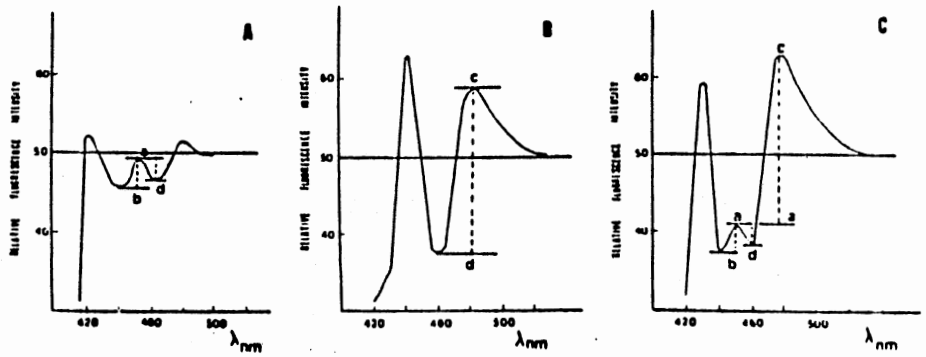
Successful application of the synchronous derivative technique for multicomponent determinations requires a difference in the excitation and/or emission maxima of the two fluorescent species. However, the difference does not have to be as large as that required for steady-state determinations. There is a significant band-narrowing effect observed when using synchronous fluorescence techniques (55), and there is further enhancement of spectral differences when the derivative signals are obtained.

The ability to resolve any two components using the synchronous derivative technique will depend primarily on the magnitude of any shift in the fluorescence excitation/emission maxima of the fluorescent species. The smaller the shift, the more difficult it will be to determine the components. For the extreme case, it would not be possible to resolve two components with complete spectral overlap.

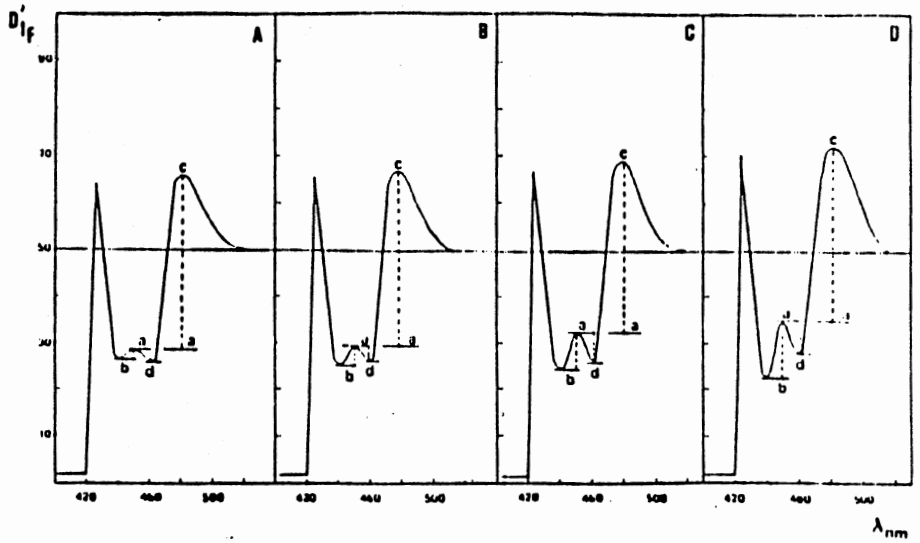
Application of the synchronous derivative technique to the simultaneous determination of gallium

and zinc using SATCH as a chelator (Figure 5) has been reported by Urena Pozo et al. (6). Studies demonstrating single component determinations of gallium (35) and zinc (42) with SATCH have been discussed earlier in this chapter. In the new study, second derivative synchronous fluorescence spectroscopy was used for the simultaneous determination of gallium and zinc. The determinations were done in a 48% aqueous ethanolic solvent. A 50 nm wavelength difference was maintained between the excitation and emission monochromators. The concentrations of zinc and gallium in solution were calculated using the peak-to-peak method (56). A sample of the measurements is given in Figure 10.

Samples of wine, drinking water, wastewater, and human urine, as well as bovine liver, kidney and brain were analyzed. The results for the determination agree quite well with the expected values: gallium concentrations from recovery studies, and zinc concentrations from atomic absorption spectroscopy (AAS). The determination of zinc was affected to a greater extent by the presence of foreign ions than the determination of gallium was. Several species, including Fe(II), Cu(II), Mo(VI), and V(V), that all interfere with the determination of zinc at a 1:1 ratio (analyte to interfering species) interfere with the determination of gallium at a 6.7:1 ratio.



Second-derivative synchronous fluorescence spectra for gallium (A) and zinc (B) complexes and for their mixture (C) at $\Delta\lambda$ = 50 nm, $\Delta\lambda'$ = 10 nm, time constant of 1.5 s, scan speed of 120 nm min^{-1}, and pH 4.7. Concentrations are 10 ng/mL for gallium and 1 μg/mL for zinc



Second-derivative fluorescence spectra of zinc and gallium complexes at different concentrations: concentration of zinc, 1 μg/mL in all cases; concentration of gallium, (A) 5 ng/mL, (B) 10 ng/mL, (C) 20 ng/mL, (D) 30 ng/mL.

Figure 10. Second derivative plots of gallium and zinc complexes of SATCH.

The method is slightly more sensitive for the determination of gallium than for zinc, but the linear range for the determination of zinc is larger than that for gallium. However, the linear range for the determinations for both metals was increased relative to the previously reported single component determinations. The linear range for the Ga-SATCH chelate using conventional fluorescence spectroscopy is 3 to 30 ng/ml, and for zinc it is 15 to 1300 ng/ml. The linear ranges using synchronous derivative spectroscopy for gallium and zinc are 2 to 40 ng/ml and 20 to 1500 ng/ml respectively.

Kinetic Methods of Determination

Although kinetic methods have also been developed for the determination of metals via chelation, the kinetic determinations are not nearly as prevalent as the steady state determinations. In a recent review of luminescent methods for metal ion determination, only 7% of the determinations were based on kinetic measurements, possibly because many experimenters do not want to introduce time as an additional source of error into their determinations (2).

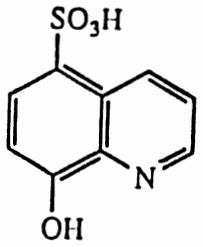
The kinetic methods that will be discussed in this section fall into two broad catagories. The first category is determinations based on the kinetics of the formation or dissociation of a fluorescent metal-

chelate complex. This type of determination can be accomplished using conventional instrumentation and, if the rate of the reaction is slow enough, require no special techniques for sample introduction. The second category is determinations based on a fluorescence lifetime difference of the metal chelates. These methods require more sophisticated instrumentation than is required for conventional measurements.

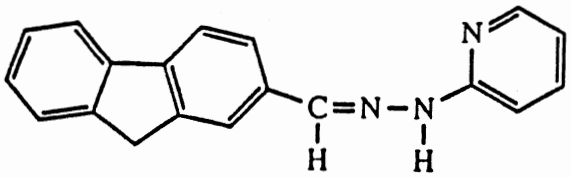
Determinations Based on Chelate Reaction Kinetics

The determinations based on reaction kinetics fall into the first category described above. Two separate cases will be discussed: one involving determinations based on the kinetics of the formation of a metal-chelate species; the other based on the kinetics for the dissociation of a fluorescent species.

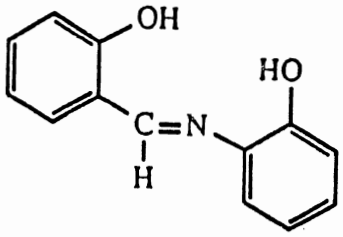
Determination of Aluminium. Aluminum was determined by examining the reaction kinetics for the formation of an aluminum chelate with 5-sulfo-8-hydroxyquinoline (QS, Figure 11) in work by Wilson and Ingle (57). The initial rate approach was used for these determinations. This approach allows the initial rate of a reaction to be linearly related to the concentration of the analyte under the proper conditions. These conditions include that the reaction



QS



FLAPH



SoAP

Figure 11. Chelators used for kinetic determinations of metals.

rate be measured within the first 5% of the reaction, insuring an approximately linear change in the reaction rate with respect to analyte concentration and minimal side reaction complications. For the determination of aluminum with QS, the rate is measured as a change in fluorescence with a change in time.

The fixed-time approach, in which the signal is measured after a fixed time interval, was used for these determinations. The time chosen for observation of the fluorescence signal was 16 seconds. This insured measurement of the initial 2% of the reaction, since the reaction took 30 minutes to reach completion. The optimum rate for the determinations occurred in the pH range of 3.0 to 3.5. Careful pH control was required for the determinations because the order of the reaction with respect to the hydrogen ion in this pH range is -2.67.

The observed rates are linear with respect to aluminum concentrations of from 0.4 to 10,000 ng/ml. This linear range is more than two orders of magnitude better than the best equilibrium method discussed. There is also no interference from either cadmium or magnesium, both of which interfere with the equilibrium determination of aluminum using QS. However, both the Cd-QS and Mg-QS complexes form very rapidly, and the initial rate measurements for the aluminum determination are not affected. However, gallium and

indium were not tested as interfering species, and both of these species form fluorescent chelates with QS at the pH for the determinations (7).

Determination of Magnesium. Magnesium was determined from measurement of the fluorescence decay from the magnesium 2-fluorenaldehyde 2-pyridylhydrazone (FLAPH, Figure 11) system in work reported by Laserna et al. (58). The magnesium concentrations were also obtained using the initial rate, fixed time approach. The time chosen for the observation of the fluorescence signal was 60 seconds. There was no indication of the time the reaction required to reach completion. The pH range for the determinations was from 12.5 to 13.3. The detection limit was not reported, however, the linear range for the determinations was 360 to 1220 ng/ml when the FLAPH concentration was 2 μM. A calibration curve over the reported linear range was given, but the only reported determination was of a 970 ng/ml sample.

The magnesium-FLAPH system initial conditions were chosen to ensure an excess of metal in the system, in contrast to every other system examined. The excess of magnesium is required because the Mg-FLAPH chelate is adsorbed onto micelles of magnesium hydroxide which exist at the working pH, and the fluorescence of the system is due to the adsorbed species. There are three

bits of evidence to support this. First, there are emission maxima at 422 nm and at 520 nm when the Mg-FLAPH system is excited at 375 nm. Earlier studies (59) show that the chelator exhibits a fluorescence maximum at 422 nm, but not at 520 nm. This indicates the band at 520 nm is due to the complex formation. Second, as the concentration of magnesium is increased enough to cause precipitation of the magnesium hydroxide, the fluorescence appears in the precipitate. Finally, when Tween 80, a substance which increases the lifetime of micelles, is added to the system the rate of disappearance of the fluorescence is slowed.

The fluorescence observed from the complex is specific for magnesium. It is possible to determine magnesium in the presence of a ten-fold excess of calcium or beryllium, and also in a twenty-five-fold excess of strontium or barium. However, there are several species which do interfere at a much lower concentration with the determinations, most notably Fe(III) and Al(III). A complete list of interfering species for this method is given in Table 10.

Determinations Based on Fluorescence Lifetime Differences

The use of fluorescence lifetime differences for the simultaneous determination of metal chelates is one that has tremendous possibilities, although at this

TABLE 10

INTERFERING SPECIES FOR THE DETERMINATION OF MAGNESIUM WITH FLAPH

Conc. Tolerated[a,b]	Species which interfere
100	Na(I), K(I), nitrate, sulfate.
50	oxalate, chloride.
25	Sr(II), Ba(II), $H_2PO_4^-$.
10	Be(II), Ca(II).
2	Ga(III)
1	Zn(II), Cd(II), In(III).
0.5	Cr(III), Fe(III), Hg(III), Al(III).
0.4	EDTA.

[a]Concentration in μg/ml.
[b]Values compared to a 0.97 μg/ml magnesium sample.

All values from Reference 57.

time very few methods have been reported in this area. With the advent of relatively inexpensive fluorescence lifetime instrumentation, the possibility exists that determinations based on chelate lifetime differences will become an important area for the development of new methods for simultaneous metal determination.

If there is a sufficiently large fluorescence lifetime difference between metal chelates, methods can be used to simultaneously determine several metals. Fluorescence lifetimes of different chelates, changing only the metal, can vary dramatically, even though the excitation and emission spectra may be quite similar or identical (7,11). Lytle et al. studied the heavy atom effect to determine the degree to which it would affect the fluorescence lifetimes for several metal complexes of 8-hydroxyquinoline (11), since it is a general chelator, and will form fluorescent complexes with many metals (60-64). The studies were the first to show that there is a possibility of determinations based on a difference in the fluorescence lifetimes of the chelates. The largest difference in these fluorescence lifetimes occurred as one progressed across a row in the periodic table; however, in many cases it was simpler to separate the metals using previously developed methods.

According to the authors, the most important application of the lifetime difference method will be

for determinations within a periodic family. It was found that there are differences in fluorescence lifetimes within a family, although for this particular ligand they may not be large enough to exploit using a time-resolved fluorimetric method. The authors suggest that the design of ligands specifically to maximize the heavy atom effect, and therefore maximize any difference in fluorescence lifetimes for the chelates of metals in a periodic family, is an important area for ligand development.

The fluorescence properties of metal chelates of salicylidene-o-aminophenol (SoAP, Figure 11) were studied by Craven and Lytle (65) to determine if it would be suitable for the simultaneous determinations of metals. Aluminum, gallium, indium, and thallium were used for these studies. The chelates were formed by refluxing the anhydrous metal chlorides with the ligand in pure ether. The precipitates which formed were subsequently dried and redissolved in a 20% methanol, 80% water solvent. The redissolved chelates were then used to study the excitation and emission spectra, and to determine the fluorescence lifetimes. The indium chelate is stable after 15 minutes in solution, and the aluminum and gallium chelates are stable after 2 hours. It was not possible to determine thallium accurately because of the instability of the

complex and the low fluorescence intensity. The results from these studies are shown in Table 11.

The excitation and emission spectra for the chelates showed only a broad, featureless peak, with a width at 1/2 height of about 200 nm, compared to the difference in the maxima for the chelates, the largest of which is 20 nm. However, the lifetimes for the chelates do show a significant difference. It has been shown (66) that a three component system can be determined if the lifetime difference ratio is 1:2.3 for the two closest fluorescence lifetimes, and 1:20.3 for the overall lifetime range. For the species shown, mixtures of any two metals except aluminum and gallium should be determinable using this algorithm, although no determinations were reported.

Hiraki et al. have developed methods for the simultaneous determination of Al(III) and Ga(III), and of Cd(II) and Mg(II), based on a difference in the fluorescence lifetimes of the metal chelates (7). For these determinations a deuterium flash lamp with a pulse width of 2 ns was used, and the resultant decay curves were deconvoluted to obtain the concentration of each metal species. The chelates formed are very weak fluorophores. The quantum yield of the most intense fluorophore, the aluminum-QS complex, was only 0.11, causing a poor sensitivity for the methods. The

TABLE 11

SPECTRAL AND LIFETIME DATA FOR SoAP CHELATES

	Al	Ga	In	Tl
excitation max.[a]	405	410	408	420
emission max.[a]	530	545	550	535
quantum yield[b]	0.34	0.20	0.04	0.01
lifetime[c,d]	5.39	2.50	0.82	<0.40

[a]wavelength values are in nm.
[b]calculated from quinine hydrogensulfate, 0.546.
[c]calculated by a graphical comparison method.
[d]units are ns.

All values from Reference 65.

results for the simultaneous determinations using QS as a chelator are shown in Table 12.

One of the obvious advantages of this system is that it allows simultaneous determination of two species. The lifetimes of the metal chelates can also be determined directly from the deconvoluted decay curves. In the case of an unknown metal species this would give valuable information as to the identity of the metal.

The reported difference in the fluorescence lifetimes for the determinations in both cases was about 10 ns, and the system apparently does not exhibit adequate resolution for the determination of species with smaller lifetime differences. The authors reported fluorescence lifetime data for species in which no multicomponent determination was reported, various combinations of which would have given lifetime differences of one to four nanoseconds.

TABLE 12

SIMULTANEOUS DETERMINATIONS OF METALS WITH 5-SULFO-8-HYDROXYQUINOLINE

For mixtures of Al and Ga.

mixture	Aluminum[a] present	Aluminum[a] found	Gallium[a] present	Gallium[a] found
1	80	84	800	731
2	200	212	200	224
3	160	148	400	400

For mixtures of Mg and Cd.

mixture	Magnesium[a] present	Magnesium[a] found	Cadmium[a] present	Cadmium[a] found
1	200	204	600	576
2	200	212	800	800
3	400	360	400	456
4	400	392	600	584

[a]Concentrations are ng/ml.

All values from Reference 7.

CHAPTER III

THEORY OF PHASE RESOLVED FLUORESCENCE SPECTROSCOPY AND DESCRIPTION OF EXPERIMENTAL PROCEDURES

Theory of PRFS

In the past few years phase-resolved fluorescence spectroscopy (PRFS) has been used for multicomponent fluorometric determinations based on fluorescence lifetime selectivity alone or in combination with other fluorometric selectivity parameters (9,67). The PRFS technique involves the use of a sinusoidally-modulated excitation beam in conjunction with phase-sensitive detection (68-70). The detailed theory of PRFS has been described elsewhere (71,72), however, a brief description of some of the important equations follows.

The excitation function for PRFS has the form:

$$E(t) = A(1 + m_{ex}\sin\omega t) \qquad (1)$$

where A is the d.c. intensity component of the exciting beam, m_{ex} is the modulation depth (the a.c. to d.c. ratio), and ω is the angular modulation frequency ($\omega = 2\pi f$ where f is the linear modulation frequency). The

resultant time-dependent emission F(t) will be both demodulated and phase shifted to an extent determined by the fluorescence lifetime of the species:

$$F(t) = A'(1 + m_{ex}m\sin(\omega t - \phi) \qquad (2)$$

where A' is the d.c. component of the fluorescence emission, ϕ is the phase shift of the species, and m is the demodulation factor for the emission (the modulation depth of the emission divided by that of the excitation). A diagram of this is shown in Figure 12.

Fluorescence lifetimes (τ) can be calculated from the phase-modulation data measured at angular modulation frequency ω by using the phase delay ϕ:

$$\tau_p = (1/\omega)\tan\phi \qquad (3)$$

or the demodulation m:

$$\tau_m = (1/\omega)((1/m^2) - 1)^{1/2}. \qquad (4)$$

In PRFS, the phase-resolved fluoresence intensity (PRFI) $F(\phi_D)$ is the integral over time of the product of the a.c. portion of the function F(t) and a periodic on-off function of the same frequency as F(t) which can be continuously adjusted to phase angles between 0^o and 360^o. The integration interval can therefore be in-phase, out-of-phase, or anywhere between these two extremes relative to the emission. The equation describing the PRFI is:

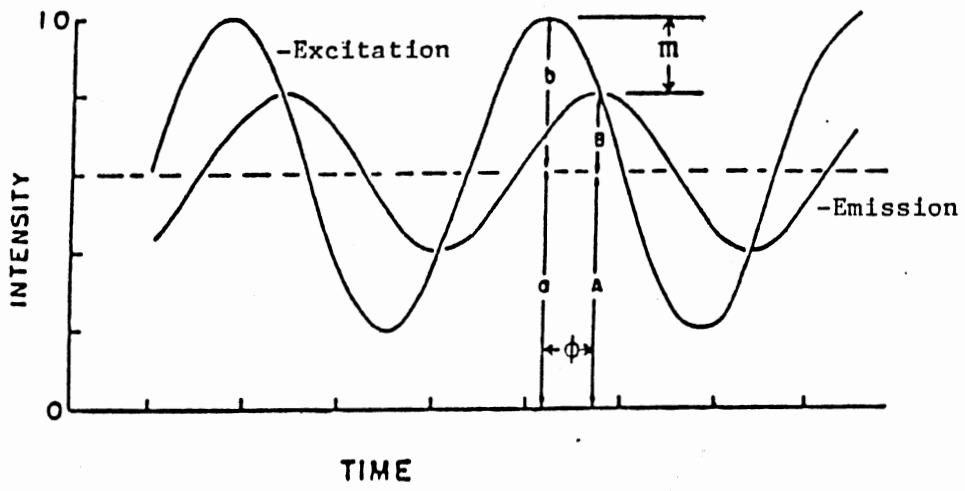


Figure 12. Diagram of the excitation and emission phase modulated signal for PRFS.

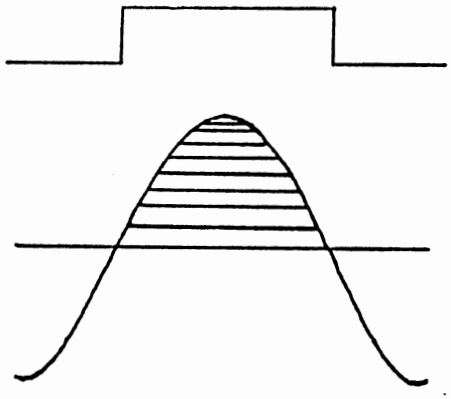
$$F(\phi_D) = A'm_{ex}m\cos(\phi_D - \phi) \qquad (5)$$

where ϕ_D is the detector phase angle setting, i.e., the position of the periodic integration function. This is shown in Figure 13.

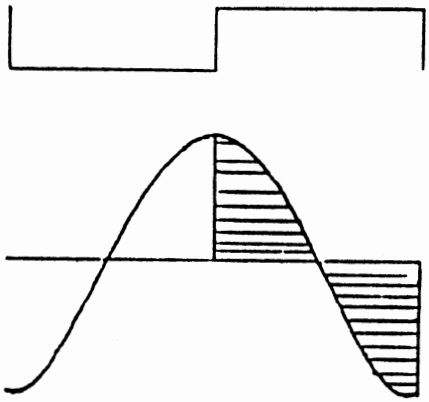
The simultaneous determination of fluorophores is accomplished by measuring the PRFI values of mixtures $(F(\phi_D)_{mix})$ and standards at different detector phase angles to generate an overdetermined series of linear equations. For a two component system the equations are of the form:

$$F(\phi_D)_{mix} = F(\phi_D)_1 + F(\phi_D)_2$$
$$= I(\phi_D)_1C_1 + I(\phi_D)_2C_2. \qquad (6)$$

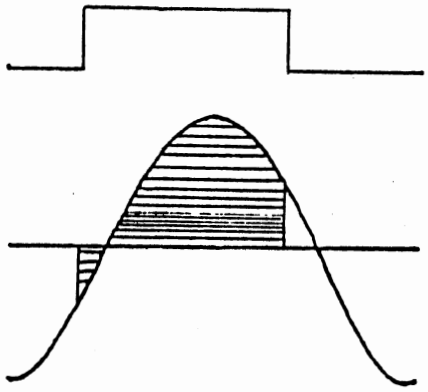
The $I(\phi_D)$ values are the PRFI values of chelates with a known concentration of a single component at each detector phase angle. These values are found from the solutions referred to as the standards. C_1 and C_2 are the concentrations of the fluorophores in the mixture. A series of linear equations, one for each detector phase angle, are solved for C_1 and C_2 with a non-negative least squares (NNLS) fitting routine (73). This approach has been previously applied to the determination of polynuclear aromatic hydrocarbons in mixtures of up to six components (8,9).



Integrating signal in-phase with emission



Integrating signal out-of-phase with emission



Intermediate setting

Figure 13. Integration function for PRFS.

Fundamental Limitations

For a particular set of conditions (excitation and emission wavelength and modulation frequency) the phase resolved approach is limited to the determination of two components. This is due entirely to the nature of the observed signal. Since the PRFS signal is a sinusoidal function, at a fixed frequency the data can be described explicitly by two parameters, the phase shift and the amplitude. For a two component system this is fine, since the observed signal is a unique combination of the two single component sinusoidal signals. However, when a third component is added another parameter is needed to completely describe the system. For the determinations of up to six components this was accomplished by changing the excitation and/or the emission wavelengths and the modulation frequency, something that was not possible with the metal systems.

The hypothesis that the system had a fundamental limitation was tested via computer simulation. A program was written to simulate a PRFS signal from lifetime and intensity data. This information, without superimposition of any noise, was then analyzed using the NNLS program. It was found that while the values for the two component system could be extracted exactly as expected, the values for the three component system varied greatly from the expected values unless data

from two modulation frequencies was combined prior to analysis. This is shown in Table 13.

All of the determinations of metal chelates were accomplished at a fixed set of excitation / emission wavelengths and a single frequency. The wavelengths were fixed because there was very little difference in the excitation/emission spectra of the chelates of different metals. Therefore, no wavelength selectivity was possible.

For most of the systems discussed in this study the fluorescence lifetimes were so short, and the lifetime differences so small, that a high modulation frequency was necessary to observe a significant phase shift. The highest frequency available on the instrument used was 30 MHz, therefore this was the frequency used for the determinations. It is because of the above conditions that these studies were limited to two component systems.

Limits of Detection and Quantitation

The limit of detection was calculated as the concentration of metal chelate required to produce a signal S:

$$S = S_b + 3s_b \qquad (7)$$

TABLE 13

NNLS ANALYSIS OF SYNTHETIC PRFS DATA

Component Number	Expected	Determined
	For lifetimes of 2 and 4 ns	
1	0.500	0.500
2	0.500	0.500
	For lifetimes of 2, 4, and 10 ns	
1	0.500	0.371
2	0.500	0.867
3	0.500	0.000
	Two phase angles; Lifetimes of 2, 4, and 10 ns	
1	0.500	0.500
2	0.500	0.500
3	0.500	0.500

where S_b is the mean blank signal for 16 measurements, and s_b is the standard deviation of those measurements. For the limit of quantitation, S was calculated as

$$S = S_b + 10s_b. \qquad (8)$$

All of the phase-resolved limits were found from phase-resolved intensity measurements at the phase angle maxima of the chelates ($\phi_D = \phi_{chelate}$). The limits, shown in Table 14, were determined in order to compare the inherent loss of signal that occurs in going from steady-state to phase-resolved measurements. Phase-resolved measurements at detector phase angles other than the maximum of the emitter will give intensities that are attenuated by a factor equal to $\sin(\phi_D - \phi_{chelate})$, thereby reducing detection and determination limits by the same factor.

The phase-resolved fluorometric detection and determination limits were approximately three and five times higher than the steady-state counterparts, respectively, for most systems tested. This is due to the inherent signal loss which occurs in the phase-resolved mode of operation. However, the two-component determinations could not be readily performed with steady-state fluorometry, and the loss of detectability is outweighed by the fluorescence lifetime selectivity that makes the simultaneous determinations possible.

TABLE 14

STEADY-STATE AND DYNAMIC DETECTION LIMITS
FOR METAL CHELATES DETERMINED

Metal	Steady-state		Phase-resolved	
	LOD	LOQ	LOD	LOQ
With 5-sulfo-8-hydroxyquinoline				
Al	12	18	36	95
Ga	16	25	48	125
In	6	12	20	50
With Lumogallion				
Al	0.8	2.5	2.5	7.9
Ga	2.4	7.2	8.3	24
With Morin				
Al	24	26	24	26
In	4	15	4	12
With SoAP				
Al	10	31	24	81
Ga	40	100	120	400

All values are in nM.

Experimental Procedures

Solution Preparation

All reagents were added to the experimental system in solution form. In many cases it was not possible to have a completely aqueous system, as some chelators were not readily soluble in distilled/deionized water. Therefore it was necessary to dissolve the chelator using a non-aqueous solvent. However, whenever possible an aqueous system was used.

Preparation of the Metals. Zinc sulfate (gold label) and cadmium sulfate (gold label) were purchased from Aldrich. The perchlorate salts of aluminum, gallium and indium were purchased from GFS Chemical. Distilled demineralized water was used for all metal solution preparations. Stock solutions of the metals were prepared by dissolution and dilution of an accurately weighed amount of the perchlorate or sulfate salt of each metal.

Preparation of the Chelators. The chelators chosen for these studies are of four distinct types, and are 5-sulfo-8-hydroxyquinoline (oxine derivative) from Aldrich, lumogallion (azo reagent), and salicylidene-o-aminophenol (Schiff's base reagent) from Pfaltz and Bauer, and morin (polyhydroxyflavone) from K and K Laboratories. The stock solutions of all

chelators were prepared daily by dissolving an accurately weighed amount of the chelator to the appropriate volume in the desired chelator solvent. Lumogallion (LG) and 5-sulfo-8-hydroxyquinoline (QS) were prepared by dissolution in distilled / deionized water. Morin stock solutions were prepared in absolute ethanol, and salicylidene-o-aminophenol (SoAP) stock solutions were prepared in spectral grade methanol.

Fluorescence Lifetime Standards. To determine the fluorescence lifetimes of the chelates it was necessary to use a fluorescence lifetime standard rather than a scattering zero-lifetime solution, since there is such a large difference between the excitation and emission maxima for the chelates. Two lifetime standards were used for this work. Scintillation grade DimethylPOPOP (1,4-bis-2-(4-methyl-5-phenyloxazol-2-yl) benzene, from Aldrich) dissolved in absolute ethanol was found to be a suitable standard for all chelators except lumogallion, for which Acridene Orange, also from Aldrich, was used. It was determined in our laboratory that DimethylPOPOP has a fluorescence lifetime of 1.45 ± 0.02 ns., and the lifetime of Acridene Orange was determined to be 3.17 ± 0.03 ns.

Solutions for Determination. Standard solutions of the metal chelates and samples for the determination of the metals were prepared in cuvette by addition of

the appropriate amounts of the metal and chelator solutions to an acetate buffer solution adjusted to the appropriate pH for the determination. Unknown solutions with varying amounts of metals were analyzed.

It was found that when the order of addition for the metal and chelator was reversed the intensity for the system varied greatly from what was expected, with a solution containing 50% of the weaker fluorophore and 50% of the more intense fluorophore exhibiting a fluorescence intensity less than the intensity for the weaker standard (Figure 14). This could be due to the formation of complexes with higher ligand:metal ratios which do not have the same fluorescence characteristics as the complexes being studied.

Data Collection and Analysis

Fluorescence measurements were made with an SLM 4800S spectrofluorometer (SLM Instruments, Inc., Urbana, IL) with a 450W xenon arc lamp source and photomultiplier tube (Hamamatsu R928) detection. Steady-state and phase-resolved fluorescence intensity measurements were made in triplicate in a ratiometric mode to compensate for possible source output and modulation fluctuations. A block diagram of the instrument is shown in Figure 15. Each intensity measurement was the average of ten samplings performed internally by the instrument over a period of

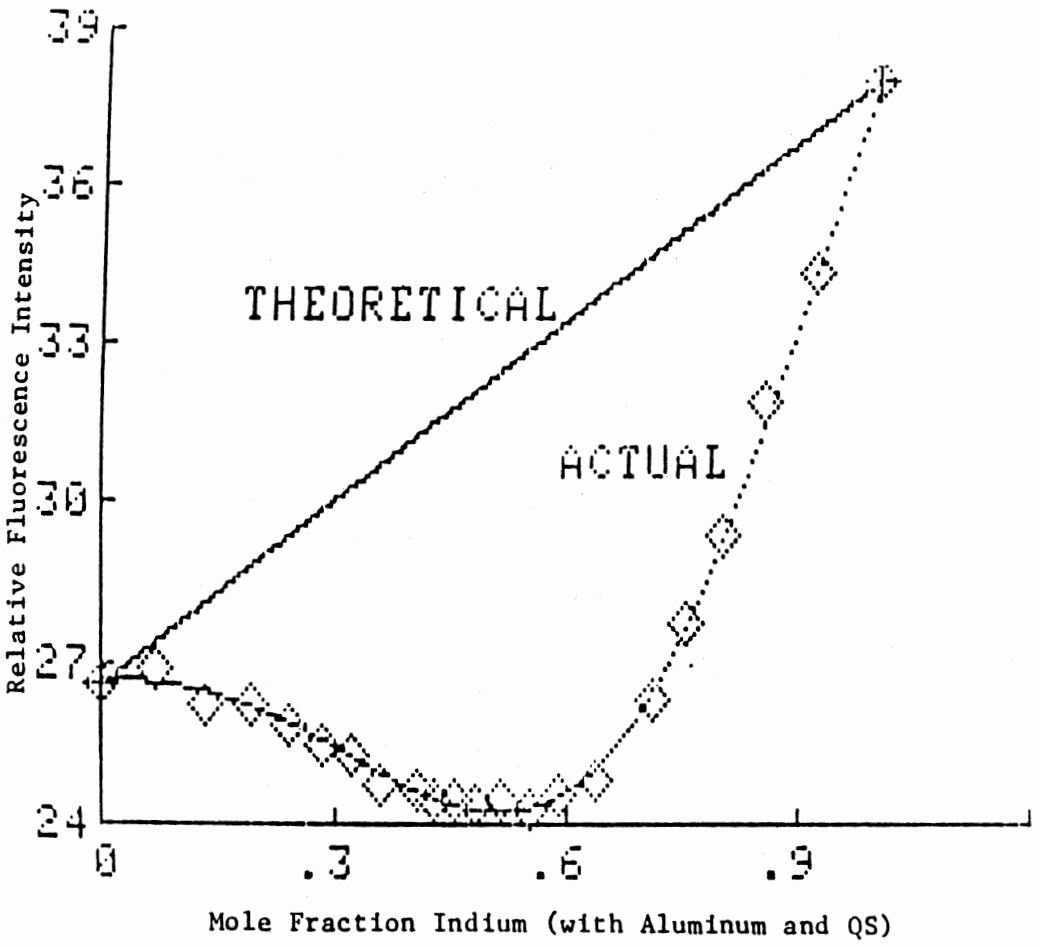


Figure 14. Mole Fraction Indium with Reverse Addition of Metal and Chelator

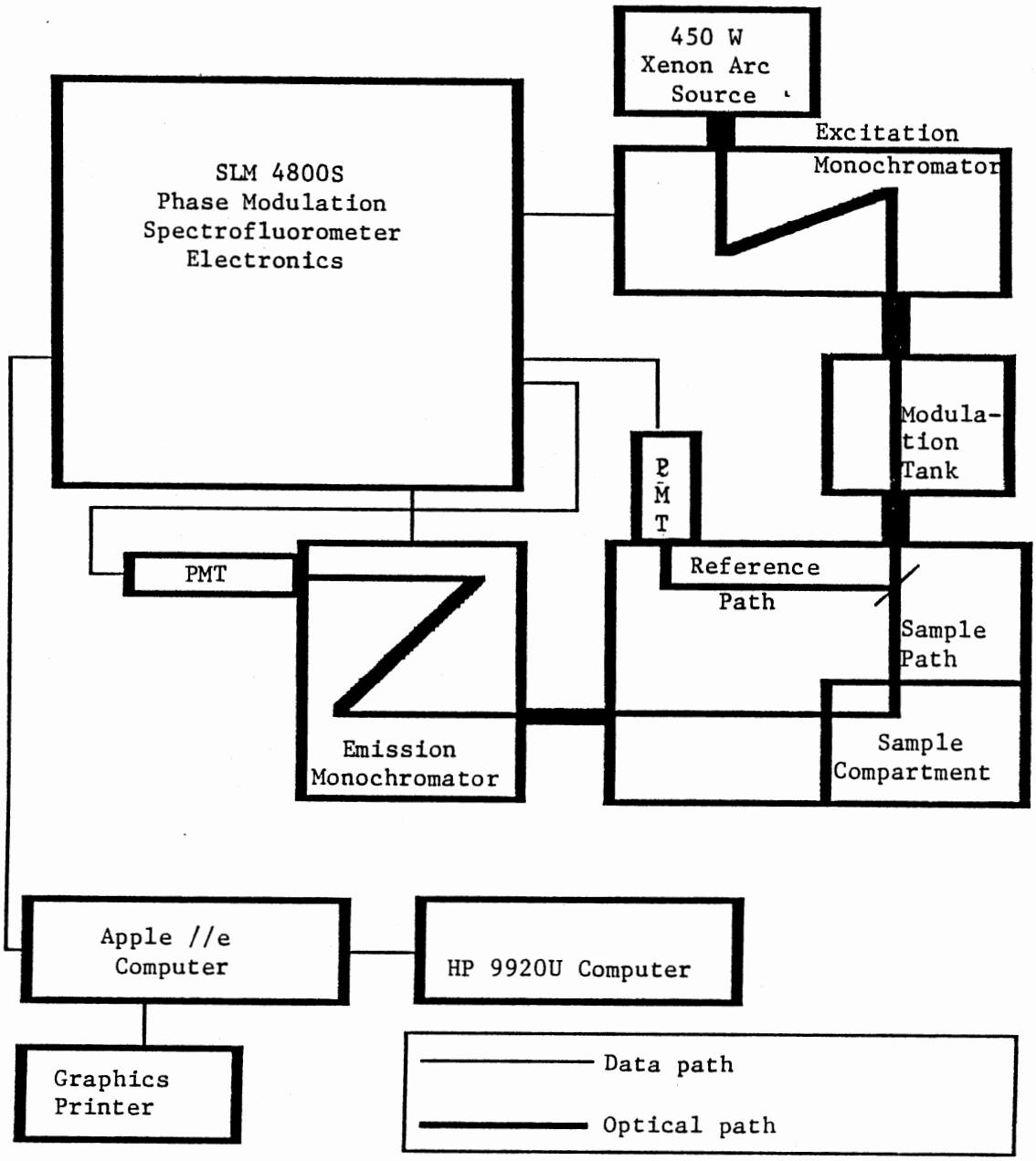


Figure 15. Block diagram of the SLM 4800S Phase Modulation Fluorometer.

approximately three seconds. Steady-state spectra for the determination of the excitation and emission maxima were acquired using both the excitation and emission monochromators. Once the wavelengths for the determinations were chosen, an interference filter was used in the emission path instead of the monochromator to increase the detected signal. The slit settings for the PRFS measurements and for the lifetime determinations were 16, 0.5, and 0.5 nm for the excitation monochromator entrance and exit and the modulation tank compartment exit, respectively. A modulation frequency of 30 MHz was used for all lifetime and PRFS measurements. The sample compartment temperature was maintained at $25.0^{\circ} \pm 0.1^{\circ}C$ with a Haake A81 temperature control unit. An APPLE IIe microcomputer with the SLM 4800S software package which was modified to include PRFS data acquisition was used for on-line data collection and lifetime calculations. The data was then dumped to an HP 9920U computer for the actual data analysis.

Two-Component Determination Procedure. The PRFI values of standards and mixtures were measured at twenty four detector phase angles for all chelators except QS, for which eight detector phase angles was sufficient in most cases. The metal standards, which consisted of a single metal in a solution of chelator

and buffer were used to find the molar phase-resolved intensity values ($I(\phi_D)$) of the chelates at each detector phase angle. The concentrations of the two metal chelates in the mixtures were found by solving the overdetermined series of equations (1 per ϕ_D), by using the NNLS routine with the HP computer through a Fortran 77 compiler. The NNLS routine does not allow negative solutions to the equations, so that negative values for the concentrations are avoided.

CHAPTER IV

RESULTS

In the following studies chelators of four general types were used for the simultaneous determination of metals based solely on a fluorescence lifetime difference. The chelators are listed in Chapter III. Five metals were determined using the chelators. The metals include aluminum, gallium, indium, zinc, and cadmium. Because not all metals formed fluorescence chelates with all chelators, determinations using all possible metal/chelator combinations were not possible. The combinations which were examined are shown in Table 15. The determinations performed with the various chelators will be discussed in the following order: lumogallion, morin, salicylidene-o-aminophenol, and 5-sulfo-8-hydroxyquinoline.

Determinations with Lumogallion

Lumogallion {5-chloro-3-[(2,4-dihydroxy-phenyl)azo]-2-hydroxybenzene sulfonic acid, (LG)} is a relatively selective chelating agent that has been used for the fluorimetric determination of both aluminum (74-76) and gallium (75,77-80). Because lumogallion

TABLE 15

METAL-LIGAND COMBINATIONS EXAMINED

	QS	LG	SoAP	Morin
Al	X	X	X	X
Cd	X			
Ga	X	X	X	
In	X			X
Zn	X			

forms fluorescent chelates with both metals, masking or a separation must be performed in order to determine one of the metals (aluminum or gallium) in the presence of the other using conventional fluorescence spectroscopy. However, a sufficiently large difference between the fluorescence lifetimes of the aluminum and gallium complexes with lumogallion exists to permit simultaneous determination of the two metal chelates based on fluorescence lifetime selectivity alone.

Fluorescence Spectra

The steady-state fluorescence excitation and emission spectra (Figure 16) of the metal-LG chelates are broad, relatively featureless, and very similar for the two metals. The excitation and emission maxima for all of the metal chelates studied are listed in Table 16.

Fluorescence Lifetimes

The fluorescence lifetimes calculated from the phase-shift are shown as a function of pH for the LG chelates of aluminum and gallium in Figure 17. The lifetimes calculated from demodulation data were within 0.1 ns of those calculated from the phase-shift method at the pH used for the determinations. The τ_p and τ_m values for all of the complexes of metals and chelators examined are shown in Table 17.

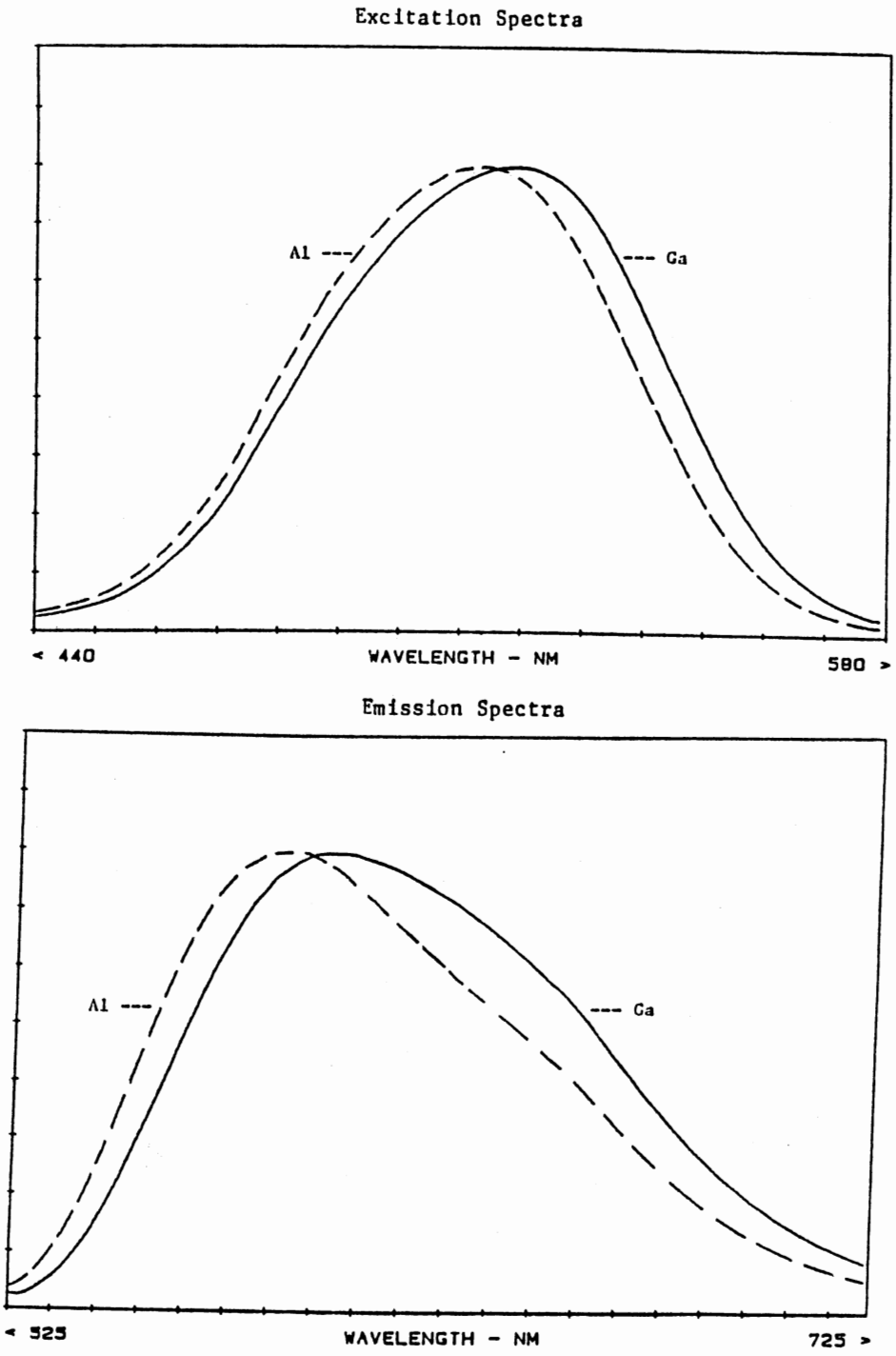


Figure 16. Excitation and Emission Spectra for the Aluminum and Gallium Chelates of Lumogallion.

TABLE 16

FLUORESCENCE CHARACTERISTICS OF THE METAL CHELATES

Metal	λ_{ex} [a]	λ_{em} [a]	intensity [b]
For LG at pH = 3.5			
Al	488	587	1.00
Ga	490	597	0.36
For Morin at pH = 3.5			
Al	408	491	1.00
In	402	491	0.31
For SoAP at pH = 5.25			
Al	400	510	1.00
Ga	400	525	0.11
For QS at pH = 4.5			
Al	367	500	1.00
Ga	370	515	0.78
In	375	520	1.01
At pH = 5.5			
Cd	373	522	0.59
Zn	374	525	1.00

[a] All wavelengths are in nm.
[b] Relative steady-state intensity for each group.

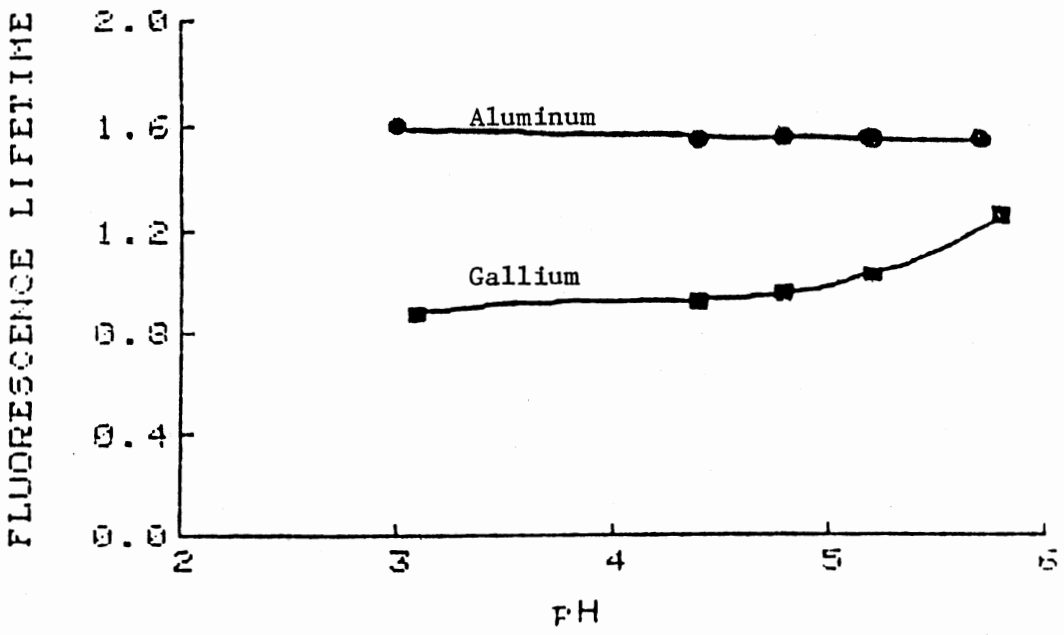


Figure 17. Dependency of the Fluorescence Lifetime on pH for the Chelates of Lumogallion.

TABLE 17

FLUORESCENCE LIFETIMES OF THE METAL CHELATES

	Phase	Modulation	Other[a]
	For LG chelates		
Al	1.56 + 0.02	1.48 + 0.008	
Ga	0.85 + 0.02	0.87 + 0.02	
	For Morin chelates		
Al	2.68 + 0.01	3.06 + 0.03	
In	0.72 + 0.01	1.38 + 0.03	
	For SoAP chelates		
Al	4.19 + 0.03	4.31 + 0.03	5.39
Ga	2.28 + 0.02	2.67 + 0.03	2.50
	For QS chelates		
Al	5.80 + 0.02	9.28 + 0.04	
Ga	0.53 + 0.003	1.18 + 0.07	
In	2.23 + 0.01	2.59 + 0.04	
Cd	3.31 + 0.03	3.73 + 0.02	
Zn	2.32 + 0.02	2.43 + 0.02	

All values are ns.

[a]From Craven and Lytle (65).

Fluorescence Intensities

The steady-state fluorescence intensities of the metal chelates as a function of pH in acetate buffer are shown in Figure 18. The intensities have a maximum in the pH range of 3.0 - 4.5, with a decrease in intensity at both low and high pH. The pH chosen for the multicomponent determinations was 3.5.

The fluorescence intensities as a function of the ratio of LG to metal are shown in Figure 19. The steady-state intensity maximum for the aluminum chelate is near 5:1, with a very gradual decrease past that point. The intensity of the gallium chelate is at a maximum at a very low ratio of ligand to metal, and is relatively constant throughout the ratio range. For the two component determinations there was at least an 8-fold excess of LG over the total metal concentration to insure a ligand excess in all cases.

Two-component Determination

A pH of 3.5 was chosen for the determinations for two reasons. First, the fluorescent lifetimes of both metal chelates are relatively constant with pH in this region, and second, the intensity for the weaker fluorophore (Ga-LG) is maximized. The phase angle difference between the complexes, which is a function

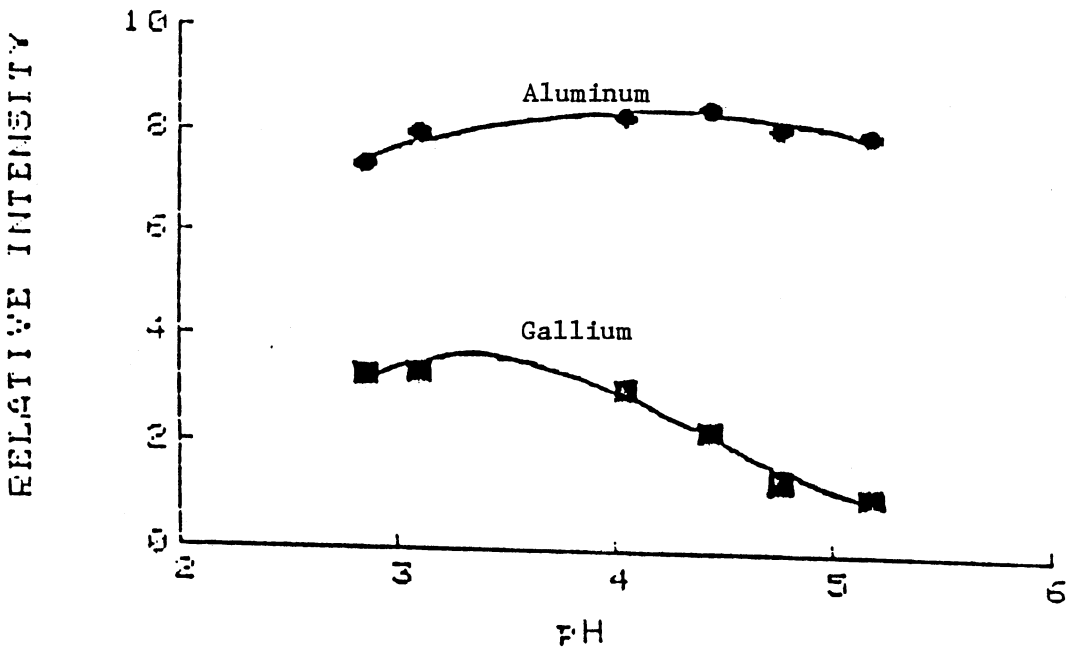


Figure 18. Fluorescence Intensity vs. pH for the Lumogallion Chelates.

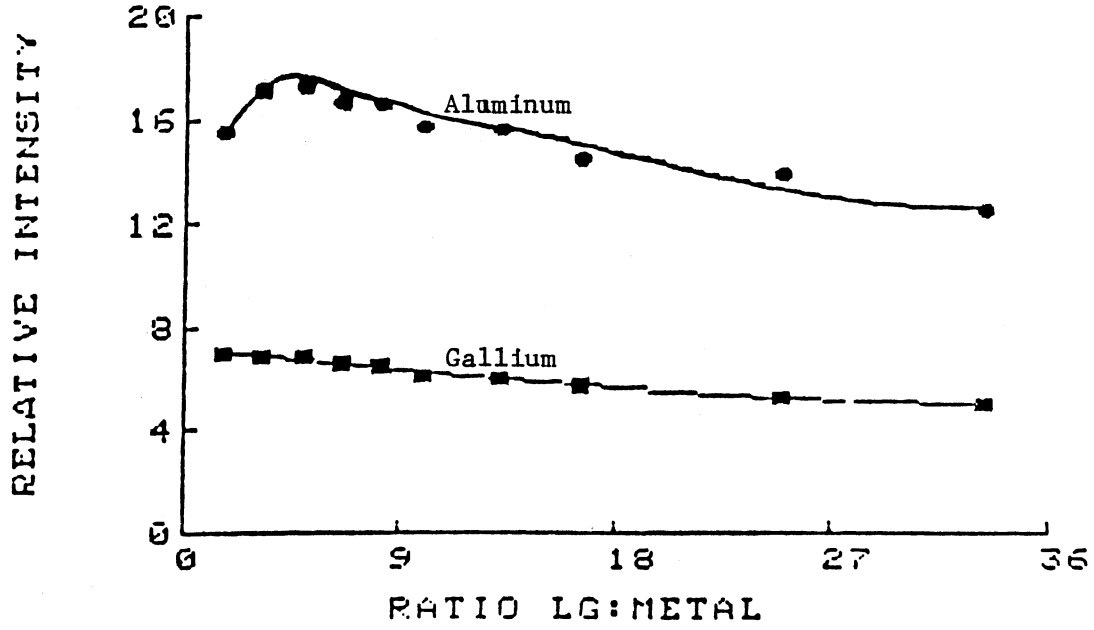


Figure 19. Fluorescence Intensity vs. Ligand Concentration for the Lumogallion Chelates.

of the fluorescence lifetime difference and the modulation frequency, is 7.5^{o} at pH 3.5.

Errors and error magnitudes for the determination of aluminum and gallium in mixtures with a total concentration of the metals of 10.0 uM were all less than 5%, as shown in Table 18. Subsequent determinations of different solutions with similar composition yielded results which were very similar to those shown in Table 18.

In this study and in all other studies error is defined as the difference between the expected value (TV) and the calculated value (CV). Percent error (%E) is given by:

$$\%E = (CV - TV) * 100\% / TV \qquad (9)$$

Results for the data set were also compiled. The reported average error is the numerical average of all %E values, and the absolute value of the average error (|Average error|) is the numerical average of the absolute values of the individual %E values.

Determinations were also done on a series of solutions in which the total metal concentration was non constant and very low. The results for these determinations are shown in Table 19. Examination of this data shows that as the concentrations of the metals are lowered, the individual errors for the determinations of the metals become larger.

TABLE 18

RESULTS FOR THE DETERMINATION OF ALUMINUM AND GALLIUM WITH LUMOGALLION

	Aluminum			Gallium		
	TV[a]	CV[b]	%E[c]	TV[a]	CV[b]	%E[c]
1	8.00	8.06	0.8	2.00	2.09	4.5
2	6.00	6.09	1.5	4.00	3.89	-2.8
3	4.00	3.98	-0.5	6.00	6.19	3.1
4	2.00	1.99	-0.5	8.00	8.19	2.4
5	1.00	1.01	1.2	9.00	9.07	0.8
Average error			0.5%			1.6%
|Average error|			0.9%			2.7%

[a]Concentration added, μM
[b]Concentration calculated, μM
[c][(CV-TV)/TV] * 100%

TABLE 19

LOW CONCENTRATION RESULTS FOR THE DETERMINATION OF ALUMINUM AND GALLIUM WITH LUMOGALLION

	Aluminum			Gallium		
	TV[a]	CV[b]	%E[c]	TV[a]	CV[b]	%E[c]
1	0.50	0.63	26	3.00	3.10	3.3
2	1.00	1.10	10	3.00	3.13	4.3
3	1.50	1.48	-1.3	5.00	5.07	1.4
4	2.00	2.07	3.5	2.00	2.26	13
5	2.00	1.86	-7.0	5.00	5.13	2.6
Average error			6.2%			4.9%
|Average error|			9.6%			4.9%

[a]Concentration added, μM
[b]Concentration calculated, μM
[c][(CV-TV)/TV] * 100%

Determinations with Morin

Historically, morin is the most significant of the four chelators because of the early work by Goppelsroeder (1) and the large volume of work published using morin as a chelator for steady-state methods (15-23), synchronous scanning derivative techniques (54), and dynamic studies (81). It was found that morin was also a suitable chelator for the simultaneous determinations of aluminum and indium based on a fluorescence lifetime difference between the metal chelates.

Fluorescence Spectra

The fluorescence excitation and emission spectra for the aluminum and indium chelates of morin were again broad, featureless peaks which were very similar to one another. These spectra are shown in Figure 20, and the excitation and emission maxima are listed in Table 16.

Fluorescence Lifetimes

Earlier work by Sawada et al. (81) has shown that when extracted from an aqueous system into isopentyl alcohol, the fluorescence lifetimes of the aluminum and indium chelates with morin are different. Also, two different ratios of metal to ligand were observed when

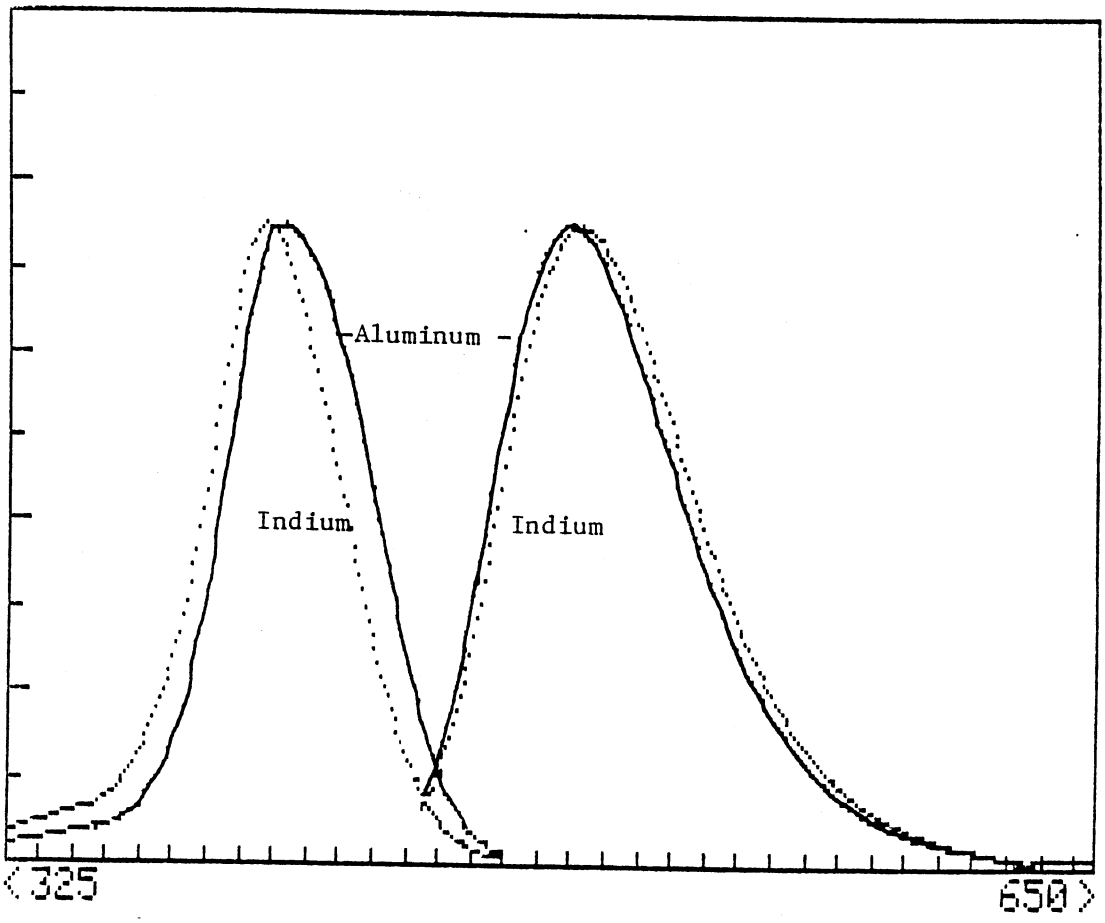


Figure 20. Excitation and Emission Spectra for the Aluminum and Indium Chelates of Morin.

extracted from aqueous solutions of differing pH; however, at about pH 3.5 there was predominantly only one ratio of ligand to metal (1:1) extracted for aluminum. It was assumed that this is probably the ratio for the indium complex also. However, this assumption was not confirmed experimentally.

It was found in this study that the lifetimes in an aqueous system also vary between the metals. The fluorescence lifetimes determined in this study are shown in Table 16.

Fluorescence Intensities

The fluorescence intensities for the aluminum chelates are consistently more intense than the chelates with the same concentration of indium. The pH dependence for the chelates is similar for both metals, with the indium chelate showing a fluorescence maximum near a pH of 3.5, while the maximum intensity for the aluminum chelate was near a pH of 4.0. The fluorescence intensity of the chelates as a function of pH is shown in Figure 21.

Two-component Determination

All determinations were performed at a pH of 3.5, since this is near the maximum intensity of the indium chelate. At this pH there is a phase angle difference of 19^{o} between the two chelates.

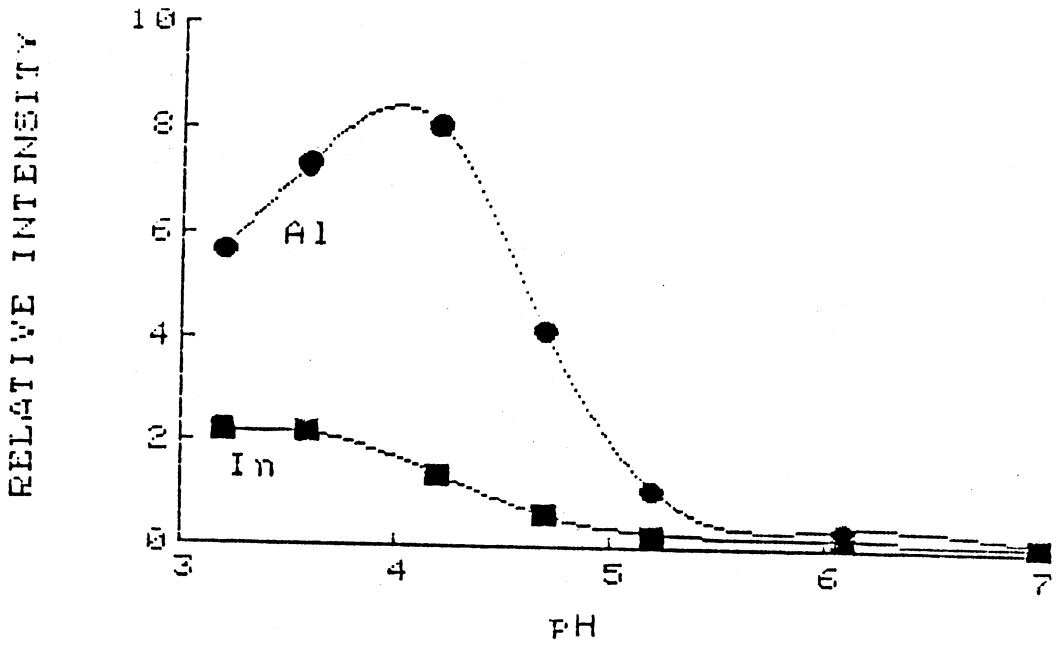


Figure 21. Fluorescence Intensity vs. pH for the Morin Chelates.

Errors and error magnitudes for the determination of aluminum and indium with morin are shown in Table 20. These values are the average for three independent determinations for mixtures of the metals with the reported concentrations. The concentration of the standard aluminum solution was 4.00 μM, while the concentration of the standard indium solution was 10.0 μM. The overall error and error magnitudes for the independent determinations are also shown in Table 20.

Determinations with Salicylidene-o-aminophenol

Salicylidene-o-aminophenol (SoAP) has been previously examined by Craven and Lytle (65) for use as a chelator for the simultaneous determination of the Group IIIA metals. In their study, the chelates were precipitated, dried, and redissolved in a 20% methanol, 80% water solvent. All measurements were then performed using the redissolved chelates.

The study by Craven and Lytle first suggested the possibility of a simultaneous determination of the Group IIIA metals, using a time-resolved approach to exploit the difference in their fluorescence lifetimes. However no such determination was ever published.

It was possible to accomplish a simultaneous multicomponent determination of aluminum and gallium

TABLE 20

RESULTS FOR THE DETERMINATION OF ALUMINUM AND INDIUM WITH MORIN

	Aluminum			Indium		
	TV[a]	CV[b]	%E[c]	TV[a]	CV[b]	%E[c]
1	3.00	3.06	2.0	2.50	2.65	6.0
2	2.40	2.39	-0.4	4.00	4.22	5.5
3	2.00	1.94	-3.0	5.00	5.26	5.2
4	1.60	1.47	-8.1	6.00	6.21	3.5
5	1.00	0.92	-8.0	7.50	7.71	2.8
			-----			-----
Average error			-3.5%			4.6%
|Average error|			4.3%			4.6%

[a]Concentration added, μM
[b]Concentration calculated, μM
[c][(CV-TV)/TV] * 100%

Individual overall results

	Aluminum		Indium	
	%E	|%E|	%E	|%E|
1	-4.6	6.6	4.2	4.2
2	-5.6	5.6	3.4	3.4
3	-0.2	2.3	6.3	6.3
	----	----	----	----
Ave	-3.5	4.3	4.6	4.6
PSD[d,e]	1.9%		1.5%	

[d]((Pooled standard deviation)/[Standard]) *100%
[e]5 samples, 3 measurements per sample.

using the phase-resolved approach to exploit the chelate lifetime difference. It was also determined that precipitation and redissolution of the chelates was not necessary.

Fluorescence Spectra

As has been the case for the ligands discussed here, the fluorescence excitation and emission spectra for the aluminum and gallium chelates with SoAP were very similarfor the two metals. These spectra are shown in Figure 22, and the excitation and emission maxima are listed in Table 16.

Fluorescence Lifetimes

Fluorescence lifetimes for the chelates of the Group IIIA elements were determined earlier by Craven and Lytle using a time-resolved method (65). These lifetimes, and the lifetimes that were determined by the phase-resolved method, are shown in Table 17. The lifetimes determined in the current study are slightly less than those lifetimes reported earlier. However, in the earlier study the solutions were all de-oxygenated prior to the lifetime determination. Since in this study de-oxygenation was not performed, one would expect the observed lifetimes to be shorter, due to oxygen quenching in the system. The results are consistent with that observation.

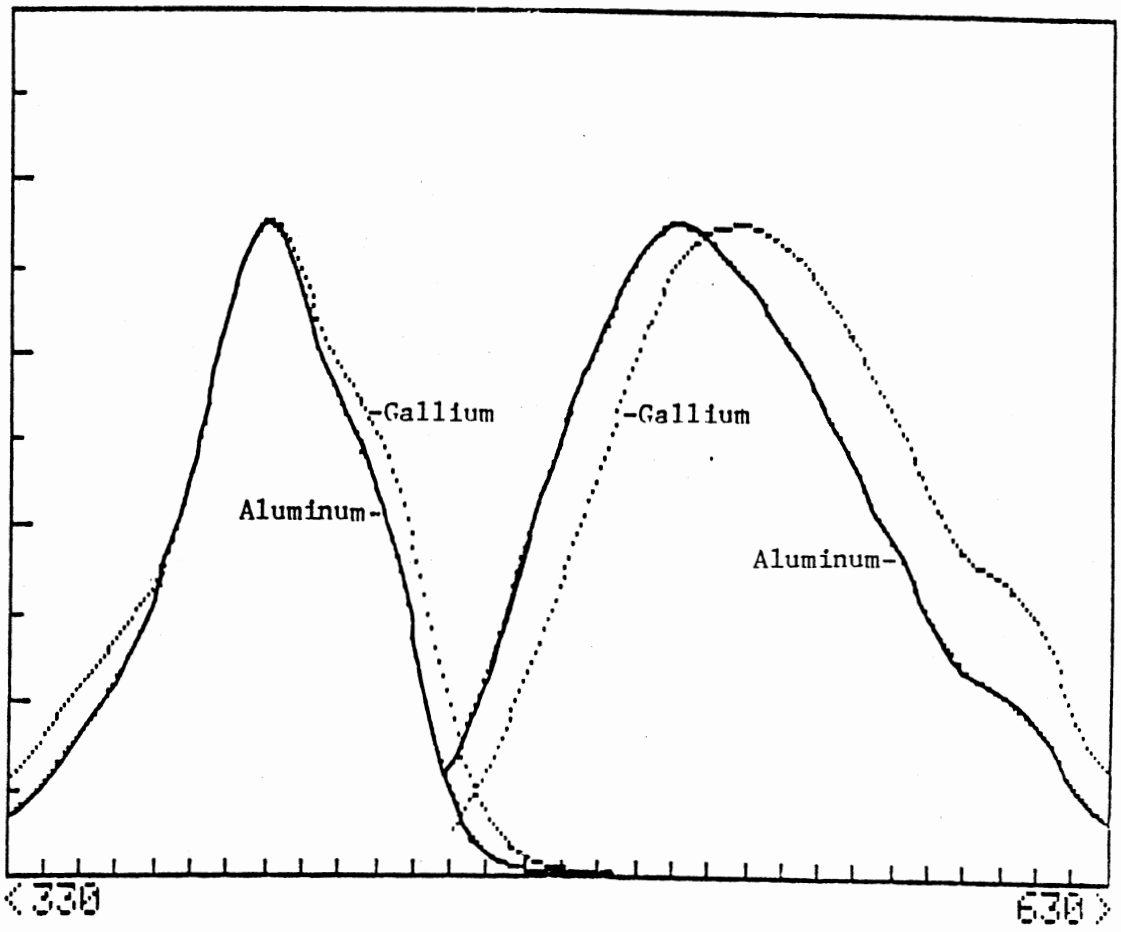


Figure 22. Excitation and Emission Spectra for the Chelates of Aluminum and Gallium with SoAP.

Fluorescence Intensities

The fluorescence intensities of aluminum and gallium exhibited quite different trends with respect to pH. While the maximum intensity observed for the aluminum chelate was near 3.7, the maximum for the gallium chelate was approximately 5.5. This is shown in Figure 23. Initially, the In-SoAP chelate was examined for fluorescence activity, however, the maximum observed fluorescence intensity for this chelate was only 3% of the aluminum signal, and was of approximately the same magnitude as the blank signal.

Two-component Determination

A simultaneous two-component determination was accomplished for aluminum and gallium using SoAP as a chelator at a pH of 5.25 for the aqueous phase, before addition of the methanol. This pH was chosen because the intensities of the 10 μM gallium and 1 μM aluminum standards were approximately equal. Three independent determinations were done over the concentration range of 0.25 - 1.00 μM for aluminum, and 2.50 - 10.0 μM for gallium.

Errors and error magnitudes for the determinations with SoAP are shown in Table 21. These values are also the average for three independent determinations of mixtures of the metals with the reported concen-

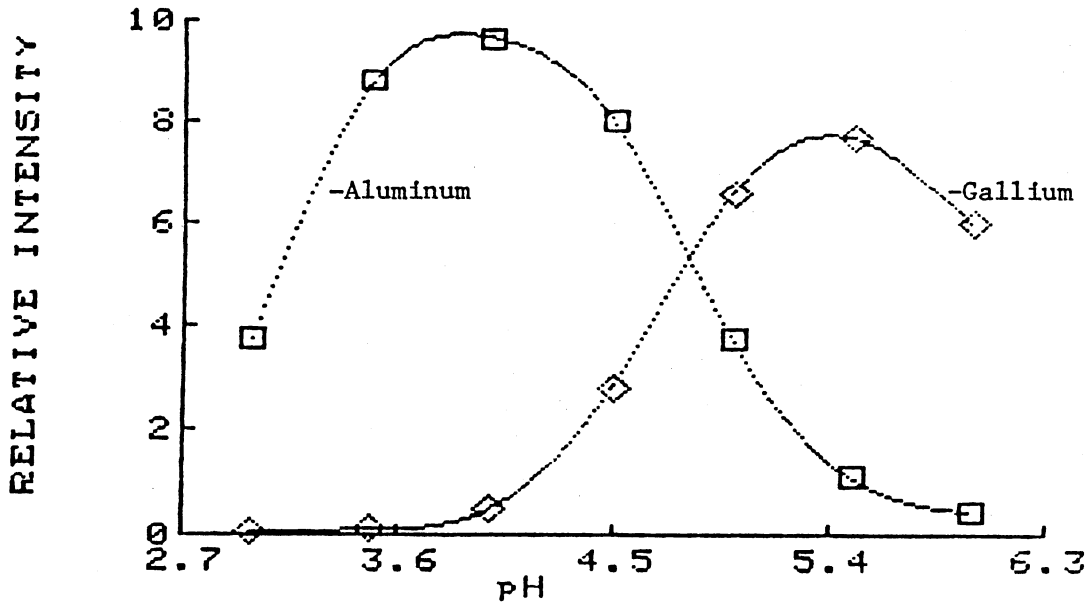


Figure 23. Fluorescence Intensity vs. pH for the Chelates of SoAP.

TABLE 21

RESULTS FOR THE DETERMINATION OF ALUMINUM AND GALLIUM WITH SoAP

	Aluminum			Gallium		
	TV[a]	CV[b]	%E[c]	TV[a]	CV[b]	%E[c]
1	0.75	0.79	5.3	2.50	2.59	3.6
2	0.60	0.64	6.7	4.00	4.02	0.5
3	0.50	0.49	-2.0	5.00	5.13	2.6
4	0.40	0.39	-2.5	6.00	6.10	1.7
5	0.25	0.22	-12	7.50	7.74	3.2
			-----			-----
Average error			0.9%			2.3%
|Average error|			5.7%			2.3%

[a]Concentration added, μM
[b]Concentration calculated, μM
[c][(CV-TV)/TV] * 100%

Individual overall results

	Aluminum		Gallium	
	%E	|%E|	%E	|%E|
1	6.9	8.5	-4.4	5.9
2	-9.0	9.0	2.9	2.9
3	1.3	4.9	8.5	8.5
	----	----	----	----
Ave	0.9	5.7	2.3	2.3
PSD[d,e]	4.1%		2.9%	

[d]((Pooled standard deviation)/[Standard]) *100%
[e]5 samples, 3 measurements per sample.

trations. The overall error and error magnitudes for the independent determinations are also shown in Table 21.

Determinations with 5-sulfo-8-hydroxyquinoline

Steady-state determinations using 5-sulfo-8-hydroxyquinoline have been reported for aluminum (57), gallium (82), indium (82), cadmium (82,83), and zinc (84). Also, a recent study by Hiraki et al. has demonstrated the use of time-resolved fluorometry for the simultaneous determination of metal 5-sulfo-8-hydroxyquinolinates (7).

This study examines the application of phase-resolved techniques to the reported determinations, and demonstrates the improvement relative to the results for the time-resolved determinations, both in precision and in number of applicable systems.

Fluorescence Spectra

The steady-state fluorescence emission and excitation spectra of the metal-QS chelates are broad, relatively featureless, and very similar for the different metals. The excitation and emission maxima for the metal chelates are listed in Table 16, and the excitation / emission spectra of the zinc and cadmium QS chelate are shown in Figure 24.

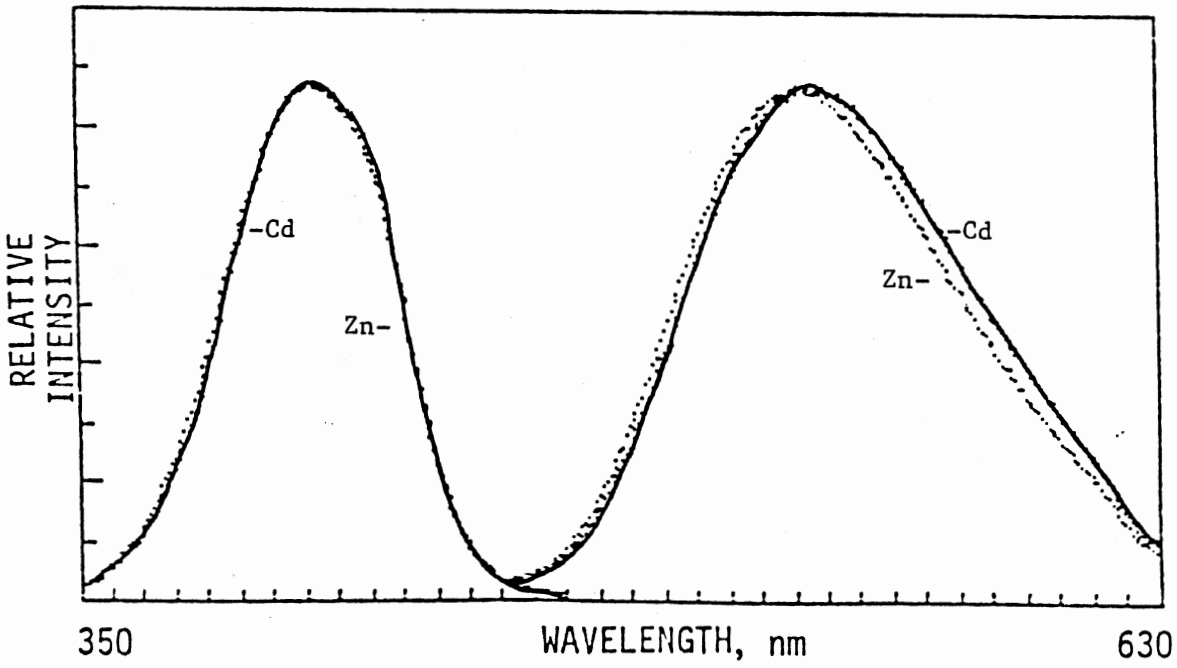


Figure 24. Excitation and Emission Spectra for the Cadmium and Zinc Chelates of 5-sulfo-8-hydroxyquinoline.

Fluorescence Lifetimes

The fluorescence lifetimes calculated from phase-shift are shown as a function of pH for the QS chelates of indium, aluminum, gallium, cadmium and zinc in Figure 25. Cd-QS intensities at pH < 5 were too low to permit lifetime determinations. The lifetimes calculated from demodulation were within 1-2 ns of those calculated from phase-shift for all of the metal complexes except Al-QS.

The phase and modulation lifetimes for the metal chelates at the pH conditions used for the two-component determinations are listed in Table 17. The τ_m value of Al-QS is much longer than the τ_p value at pH 4.5 (Table 17), indicating ground state heterogeneity, i.e., the presence of more than one fluorescent component. At both lower and higher pH values, the phase and modulation lifetimes tend to converge (at pH 3.21: τ_p = 8.37±0.51 ns and τ_m = 9.94±0.17 ns; at pH 7.00: τ_p = 1.04±0.02 ns and τ_m = 2.14+0.11 ns). Therefore, the heterogeneity at pH 4.5 indicates a mixture of the longer-lived low pH component and the shorter-lived high pH component.

Fluorescence Intensities

The steady-state fluorescence intensities of the QS chelates as a function of pH in acetate buffer are

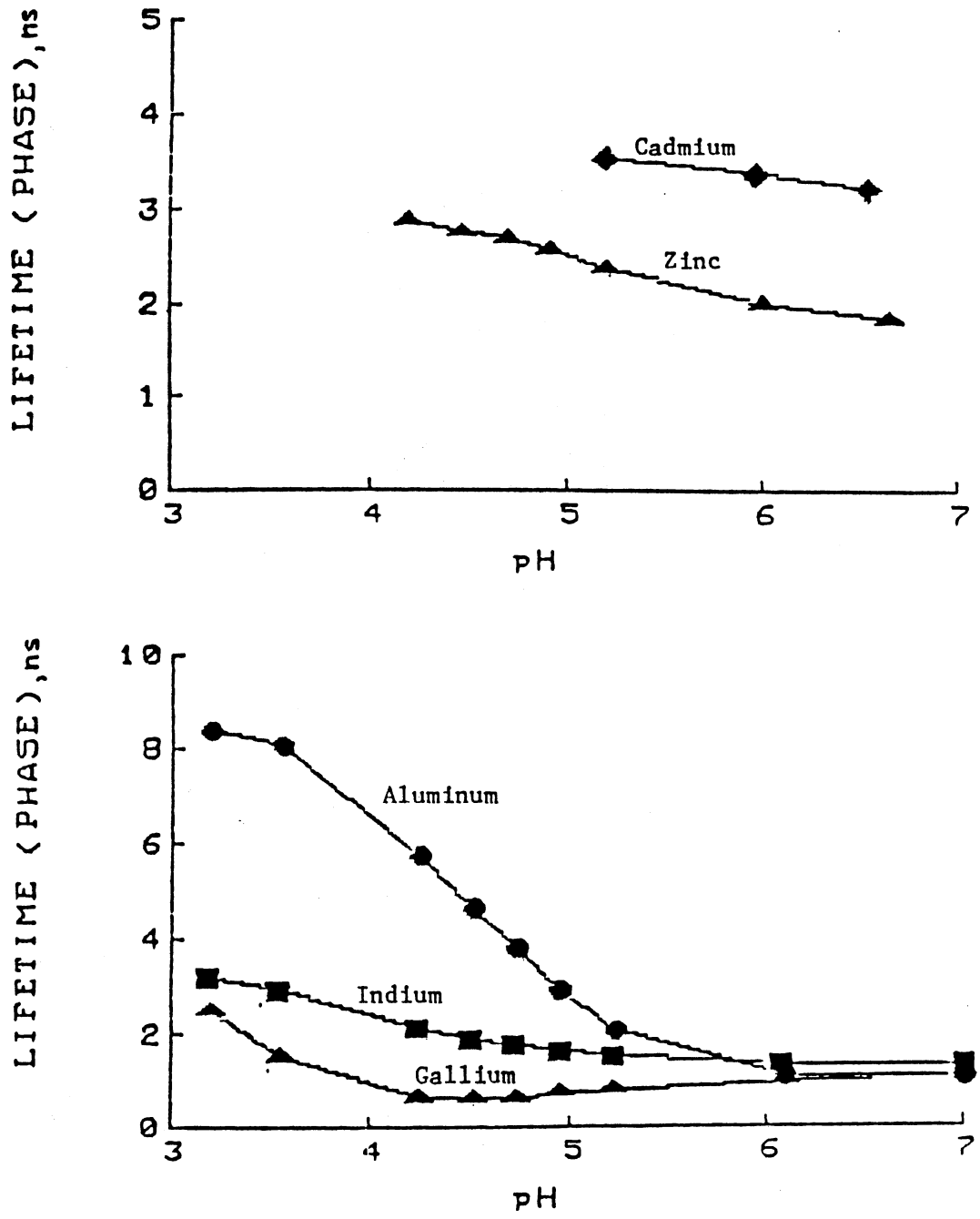


Figure 25. Fluorescence Lifetime vs. pH for the Chelates of QS.

shown for aluminum, gallium, indium, zinc, and cadmium in Figure 26. The intensities increase with increasing pH except for the Al-QS chelate which has a peak intensity near pH 4.2. The pH effect on intensity is especially pronounced for zinc and cadmium, for which the intensities are very close to zero at low pHs.

The fluorescence intensities as a function of the analytical concentration ratio of QS to metal were found to level off at QS:metal ratios in the range of 2 to 4. For the determination of metals in two-component mixtures, the concentration of QS was ten times the total metal concentration to ensure an excess of the ligand in all cases.

Two-Component Determinations

The pH used for each two-component system was chosen to effectively combine three factors, including maximum fluorescence lifetime difference between the components, high intensity and buffering capacity. For Ga/In and Al/Ga mixtures, pH 4.5 was used as a compromise between large lifetime differences at low pH and high intensities at high pH. The Zn/Cd mixtures were analyzed at pH 5.5, sacrificing intensity somewhat in order to remain in a good buffering range for the acetate buffer. The lifetime difference between the zinc and cadmium chelates is relatively constant over the pH range studied. Phosphate buffer was also used

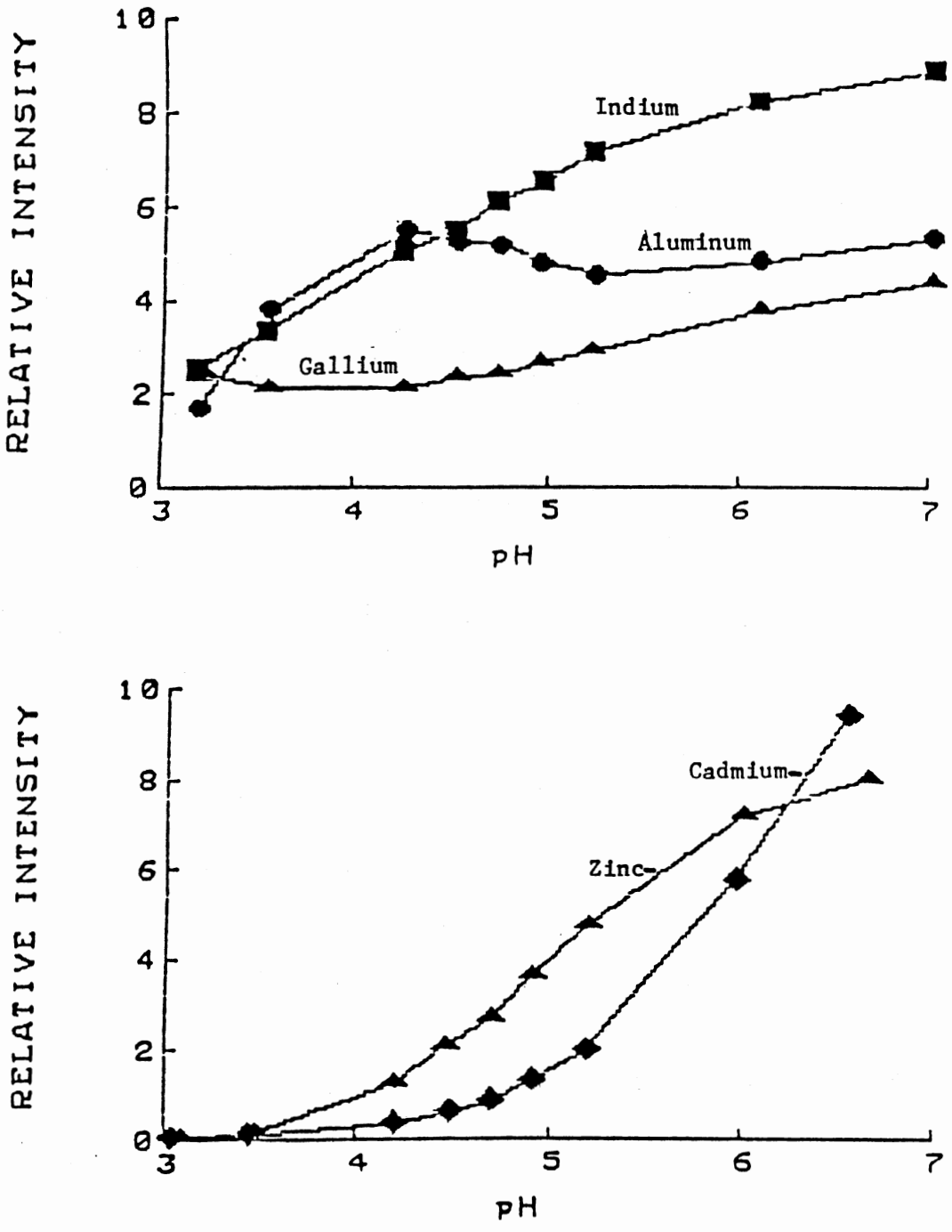


Figure 26. Fluorescence Intensity vs. pH for the Chelates of QS.

in early studies to broaden the pH range, but complexation was very sluggish and determination results were much poorer for the phosphate systems. Therefore, acetate was chosen as the buffer for the final determinations.

Errors and error magnitudes are shown in Table 22 for the determination of two-component mixtures of zinc and cadmium, indium and gallium, and aluminum and gallium. The error values shown are the averages of five mixtures for each system. The phase angle differences, which are a function of the fluorescence lifetime differences between the two components and the modulation frequency used, are also given. The average determination error found for aluminum and gallium is very similar to that reported by Hiraki, et al. (7) for the same concentration range, although the average error magnitude for their determinations was approximately three times larger. They did not report simultaneous determinations of Zn/Cd and Ga/In, which have smaller lifetime differences, possibly due to the time-resolution limitations of their instrument.

The results shown in Table 22 were obtained by using the raw (non-smoothed) phase-resolved intensity data. No significant improvement was observed when the intensity values were taken from curves fit with a least-squares cosine fit. Determinations were also accomplished using sixteen and twenty four detector

TABLE 22

AVERAGE ERRORS AND ERROR MAGNITUDES FOR THE DETERMINATION OF TWO-COMPONENT MIXTURES OF METALS WITH QS

System	%E	|%E|	Phase Angle Shift
Zn/Cd:			
Zn	2.1	2.6	11°
Cd	-1.9	3.3	
Ga/In:			
Ga	-4.1	4.3	19°
In	-1.4	1.5	
Al/Ga:			
Al	-0.9	1.0	44°
Ga	-1.5	2.1	

Average relative errors (%E) and error magnitudes (|%E|) for the determination of each component in five mixtures per two-component system.

phase angles. Again, no significant improvement was observed when this was done.

Comparison to Other Techniques

The results obtained for the detection limits of the species examined were compared to other techniques for trace metal determination. These results are shown in Table 23. Also shown in Table 23 are the lowest values actually determined for the species in a simultaneous determination using PRFS.

For several of the determinations, it is shown that the results from the PRFS technique are comparable to those from other techniques, and that the theoretical detection limits are in most near the limits for the other methods. Also, the PRFS technique is a simultaneous determination method, while AA cannot be, and ICP can be a truly simultaneous technique only with the use of a polychromator and multiple detectors.

The interferences that arise from the use of the fluorescence methods and flame spectroscopic methods are quite different. The interferences that arise in the atomic techniques occur mainly because of the high temperature processes that can affect the signal from the analyte and the relative ability or inability to introduce the matrix in which the analyte resides into the flame or plasma. The interferences from fluorescence determinations, however, are mainly due to

TABLE 23

COMPARISON OF LIMITS OF DETECTION FOR PRFS, ATOMIC ABSORPTION, AND ICP

	PRFS			
	LOD	Demonstrated	AA[a]	ICP[a]
Al	0.07	7	20	0.2
Cd	------	0.1	0.5	0.07
Ga	0.6	0.07	50	0.6
In	0.5	0.2	10	0.4
Zn	------	0.1	1	0.04

[a]From Reference 85
Values are ng/ml

problems that arise in solution chemistry, such as spectral overlap, fluorescence quenching, and non selectivity of the chelators.

Phase-resolved determination techniques are best considered as an alternative determination technique, rather than a 'new' method to replace the old. The molecular and atomic methods should be considered as complimentary rather than competitive methods. The choice of method for the determination of a metal would then be mainly dependent upon the composition of the matrix in which the species resides, and not because of an inherent advantage of one technique over another.

CHAPTER V

CONCLUSIONS

The application of PRFS techniques to the simultaneous determination of metals has the potential to become an important advance in fluorescence methods for the determination of trace metals. Although there are limitations as to the type of metal that can be determined, these techniques are somewhat complimentary to the traditional methods (AA and ICP) for trace metal determinations.

Of the four chelators examined, there are several conclusions that can be drawn. All of the chelators had both good and bad points, and these will be examined in this chapter. Future directions that this type of determination can take will also be discussed.

The most promising chelator for multicomponent determinations is 5-sulfo-8-hydroxyquinoline. This was by far the most general chelator, forming fluorescent species with all of the metals examined. It is also reported that many other metals which were not examined in this dissertation also form fluorescent species with QS (2). The lifetime differences between the chelates in some cases (Al and Ga; Cd and Mg) (7) were the

largest lifetime differences for any of the chelators examined, and lead to the largest shifts in the phase angle maximum observed in these studies (44^{o} for the Al/Ga system).

The chelates were quite stable, which is important because of the time required to complete a determination using this technique. Also, because of the large phase angle shifts and the stability of the chelates, it was possible to determine the species using only eight detector phase angles. This allows the determination to be accomplished in much less time relative to the other determinations, which required the use of twenty four detector phase angles for adequate results.

The problems with QS stem from the low quantum yields of the chelates. The intensities from these chelates were the lowest of any of the chelators examined. Although in some cases it was possible to determine lower concentrations of metals with QS than with other more intense chelators, because of the recent introduction of instruments capable of generating much higher modulation frequencies, it should be possible to lower the determinable concentration of metals with the other chelators by increasing the modulation frequency. This frequency increase will increase the phase angle shift between the chelates which have shorter lifetimes and smaller

lifetime differences, thereby increasing the resolution for the species, and lowering the minimum determinable concentration.

Lumogallion has the potential to become a powerful tool for the determination of mixtures of aluminum and gallium. The fluorescence from the chelates of lumogallion are very intense, and the chelates are essentially homogeneous, as determined by comparison of the phase and modulation lifetimes. Although the phase angle shift was quite small (7.5^{o}) this can be increased by using a higher modulation frequency. The optimum frequency for the determination of these species is about 100 MHz, and since instrumentation is now commercially available to accomplish modulation at this frequency, the minimum determinable quantity could in all probability be lowered.

A disadvantage of the lumogallion system is that as presented, the method is limited to the determination of mixtures containing aluminum and gallium only. It may be possible to increase the number of metals that can be used with this ligand by changing the solvent and/or the pH conditions.

The use of morin as a chelating agent was also limited to two components in this study. However, there is a large body of work presented in the literature using morin for the determination of several other metals. One metal that forms fluorescent

chelates with morin and which has been mentioned previously in this dissertation is beryllium (17,54). If investigation of beryllium and other metals demonstrates that there is a unique fluorescence lifetime for each, the PRFS technique could probably be applied successfully to the simultaneous determination of other metals, assuming the proper conditions (pH and solvent) would be suitable for more than one component.

A problem with morin is that it is not soluble to a suitable extent in water. This lead to the use of a non-aqueous solvent system for the determinations. With the concentrations of metal and percentages of ethanol in the solvent there were no problem with the determinations presented in this dissertation. However, if the concentration of ethanol is increased, special precautions may have to be considered to minimize solvent evaporation. The reagent also has to be fairly pure. Determinations that were attempted with non reagent grade morin without purification were not successful.

The final chelator used was SoAP. Of the four chelators discussed, this was the least promising. The solutions as prepared were very unstable, and also required about 30 s to reach a photoequilibrium. There was also a slight photodegradation with time. Because this method does require a relatively long time to complete the measurement, the degredation hampered the

determinations somewhat. Also, SoAP was limited to the determination of only aluminum and gallium, and the solvent system was 20% methanol. Given a choice of chelators for the determination of aluminum and gallium, lumogallion is a much better choice.

However, in spite of the drawbacks with SoAP, it allowed a multicomponent determination with the lowest concentration of aluminum. It may be possible to change the solvent and/or pH such that the chelates are more stable, increasing the suitability of SoAP as a ligand for multicomponent determinations.

Future Directions

There are several developments that can be examined to improve the usefulness of the application of PRFS to the determination of metals as described in this dissertation. The most obvious one is the application of this method to real samples. All determinations presented here are for laboratory-prepared 'synthetic' unknowns. If this method is to be viable, application to the analysis of a real sample needs to be demonstrated.

A second area which could be explored is the determination of a single component in a mixture of metals (such as aluminum in a mixture with gallium and indium). Many times a determination of all components in a mixture is not necessary. Since the PRFS signal

due to all interfering species can be described mathematically as a single sinusoidal function, if that function can somehow be described explicitly, the signal due to the analyte could then be determined, and a single component determination would be possible.

The PRFS technique has been applied successfully to the determination of more than two fluorescent components in a mixture, and it has been shown through computer modeling that three components with different fluorescence lifetimes can be resolved using a minimum of two frequencies, without the use of wavelength selectivity. With the introduction of instrumentation like the SLM 48000S, which is capable of continuous frequency modulation from 1 to 250 MHz, rather than the 3 fixed frequencies (6, 18, and 30 MHz) available with the SLM 4800S, a simultaneous determination of three or more metal chelates should be possible using several different modulation frequencies.

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