REVERSED-PHASE TLC AND EUROPIUM-SENSITIZED FLUORESCENCE FOR THE DETECTION AND DETERMINATION OF DIPHACINONE

By

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The objective of this study was to detect and quantitatively determine the amount of diphancinone (2-diphenylacety-1,3- Indandione) in the commercial rat and mouse bait from Durvet using reversed-phase TLC via europium-sensitized fluorescence.

The study was conducted under the guidance and help of Dr. Louis P. Varga who served as major adviser. Appreciation is also extended to Dr. E. J. Eisenbraun and Dr. H. A. Mottola as the other members of the advisory committee.

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CHAPTER I

INTRODUCTION

The combination of in situ fluorometry and thin-layer chromatography (TLC) is recognized as a valuable analytical technique for the evaluation of pollutants. Ragab (1) was probably one of the first to use fluorogenic chelate spray reagents for the detection of organothiophosphorous pesticides on thin-layer chromatograms. This work led to the development of other chelate spray reagents such as a palladium-fluorescein combination for the quantitative evaluation of organothiophosphorous pesticides by Bidleman et al, (2) and the utilization of Flavones as fluorogenic spray reagents by Mallet and Frei (3).

The current tendency is to measure florescence derived from the pesticide to be determined through its natural fluorescence or from a process of conversion. Either case is advantageous since each fluorescing species is characterized by its own absorption and emission spectra, which is not the case when fluorescence is produced indirectly such as with fluorogenic spray reagents. Detection of naturally fluorescent pesticides such as

benomyl, coumatetralyl, diphacinone and others have been measured directly from silica gel thin-layer chromatograms by Mallet et al (4).

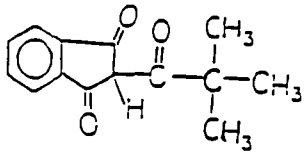
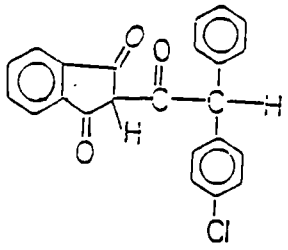
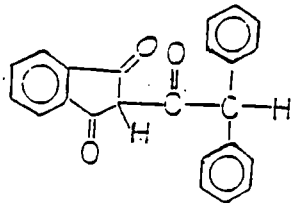
Diphacinone [2-diphenylacetyl-1,3-indandione], Chlorophacinone [2-{2-(4-chlorophenyl}-2-phenylacety)-1, 3-indandione] and Pindone [2-pivaloyl-1,3-indandione] are anticoagulants with LD_{50} values of 2.3 mg kg^{-1}, 20.5 mg kg^{-1} and 50 mg kg^{-1}, respectively (5). (See Table I). They are supplied as oil concentrates, tracking powders, grain-mix baits, pellets and wax-bait blocks. The use of this class of pesticide had led to a requirement for suitable analytical methods for the investigation of suspected poisonings in non-target species arising from direct ingestion of baits or caused by secondary poisoning through ingestion of poisoned rodents (6).

There are several published methods for the determination of these compounds in bait materials and biological media (6,7,8,9,10). The methods that are published include HPLC, ion-pair HPLC, reversed-phase ion pair LC and thin-layer chromatography. It was the goal of this study to quantitatively determine Diphacinone from extracted rat bait by using a combination of reversed-phase TLC and europium-sensitized fluorescence using the fact that Diphacinone, having a ß-diketone structure, will form fluorescence complexes with europium (III) (11,12,13). In this study diphacinones from the

standards and those separated from the extract were scraped off the plate, treated with europium (III), diluted, and the fluorescence emission read with good sensitivity.

TABLE I

STRUCTURES, NAMES AND ACTIVITIES OF ANTICOAGULANTS

Anticoagulant	Common Chemical Name	Activity
	Pindone 2-Pivaloyl-1,3-Indandione	LD_{50} 50 mg kg^{-1}
	Chlorophacinone 2-[2-(4-Chloro phenyl)-2-phenylacetyl] indan-1,3-dione	LD_{50} 20.5 mg kg^{-1}
	Diphacinone 2-Diphenyl acetyl-1,3-Indandione	LD_{50} 2.3 mg kg^{-1}

CHAPTER II

THEORY

Until the middle of this twentieth century, the separation of a sample into its various components was accomplished in painfully slow ways, such as paper or column chromatography. Beginning with the advent of practical gas chromtography in the 1950's, high efficiency in sample separation became common place. After the pioneering work of Justus Kirchner and Egon Stahl, TLC became important for the separation of samples not amenable to analysis by gas chromatography (14).

TLC is a solid-liquid-partitioning technique. The moving liquid phase is allowed to ascend a thin layer of adsorbent coated onto a backing support. When a thin-layer plate is place upright in a container with a shallow-layer of solvent, the solvent will ascend the layer of adsorbent on the plate by capillary action (15). In general TLC is a method of micro-adsorption chromatogrophy in which the sample is continually fractionated as it migrates through an adsorbent layer with the aid of a suitable solvent.

Between 1970 and 1979, TLC held second place behind liquid chromatography with a total of 27% of all chromatograpic publications being devoted to TLC. Macek attributed the popularity of TLC to the modest demands on instrumentation and its sensitivity, general applicability, and flexibility (16). High activity was observed in recent years in the areas of chemically bonded reversed-phased (RP) TLC, high performance (HP) TLC and quantification by densitometric scanning. Automation of various steps in the TLC process, most notably sample application and data acquisition and interpretation is the current center of interest in TLC research (16).

TLC is the workhorse of the pharmaceutical industry for determining drug purity and it is widely used in medical laboratories and in the chemical industry. It is one of the most frequent methods used to screen urine for illegal drugs. Besides being inexpensive, i.e. if one considers only the cost of chamber plates, micropipets for manual application of samples and the small solvent usage, TLC allows simultaneous screening for several drugs at the same time. Inherent accuracy is another additional advantage because of the ability to run samples and standards on the same plate under identical conditions. For some classes of drugs, TLC is the only screening technique that will separate closely related compounds (17) due to the wide variety of development techniques that allow increased resolution of complex mixtures

and the numerous universal and selective detection procedures possible because of the static nature of visualization process (16).

Recent Improvements in TLC

Two of the major improvements in TLC upgrading its efficiency and precision, are HPTLC and chemically bonded RPTLC. Recent publications on separation techniques using these two types of chromatographic separations include determination of phthalate esters in water by solid phase extraction and quantitative HPTLC (18), enantiomeric separation of amino acids by one and two-dimensional TLC using reversed-phase HPTLC plates (19), RPTLC of carotenoids (20), and ion-pair RPTLC of organic acids, to re-investigate of the effects of solvent and pH on R_F values (21).

High Performance TLC Plates

HPTLC is characterized by use of glass plates precoated with a silica gel layer consisting of an extremely dense packing of small particles of very uniform size with a smooth, homogeneouse surface, as well as refined, optimized techniques for all steps of the TLC procedures such as sample application, plate development, zone detection, and quantitation. HPTLC had the ability to separate nanogram (10^{-9}) to picogram (10^{-12})

quantities of analytes by rapid development (usually <10 minutes) over short migration distances (3-7 cm). Because of decreased band broadening, compact zones are produced, leading to high detection sensitivity, resolution and efficiency. Smaller samples are used so that a large number of samples per plate can be applied. Table II lists a comparison of typical results of conventional TLC and HPTLC (22).

Studies by Guiochon (23) indicated that the theoretical plate height increase (efficiency decreases) very rapidly with increasing length of run in HPTLC, while the increase in HETP is much slower with larger particle conventional thin-layer. This leads to the conclusion that short development distance are mandatory in HPTLC for highest efficiency, especially with relatively small molecules having large diffusion coefficients (22). This has been confirmed experimentally by Brinkman et al (24). These workers reported a value of 20 μm for a minimum plate height in HPTLC. Separation distance of 3-7 cm are generally recommended for highest efficiency per time of development in HPTLC (See Table III) (25). These data indicate that efficiency (N) is, in general, directly proportional to development distance between 3 and 7.8 cm. However, the number of theoretical plates per cm decreases with increased development distance. Thus, the highest

TABLE II

COMPARISON OF TLC AND HPTLC

	TLC	HPTLC
Plate size	20 X 20 cm	10 X 10 cm
Sample volume (capillary application)	1-5 μl	0.1-0.2 μl
Diameter of spots	3-6 mm	1.0 mm
Diameter of separated spots	6-15 mm	2-5 mm
Solvent migration	10-15 mm	3-6 mm
Detection limits		
Absorption	~ 5. . ng	~ 0.5 ng
Fluorescence	~ 0.1 ng	~ 0.1 ng

TABLE III

EFFECT OF DEVELOPING DISTANCE ON EFFICIENCY FOR HPTLC PLATES

Development distance (cm)	Development time (min)	N	N/cm
3	4	1183	394
4	7	1267	296
5	9.5	1076	161
6	14	1384	151
7.8	18	1600	115

Where $N = 16 \left(\frac{t_R}{W}\right)^2$

N = Efficiency (theoretical plates)
t_R = Compound migration distance
W = band width (size in direction of development)

efficiency per cm was found at the shortest distance (22).

The major advantage of HPTLC as compared to other chromatographic methods (e.g. HPLC, GC) is the speed of analysis on a per sample basis. This is due to the ability to spot multiple samples on a single plate, the short development distance and resultant short development time. Spotting samples along with standards on the same plate allows them to be processed under identical conditions (in contrast to sequential analysis on a column) for maximum accuracy and reproducibility.

Reversed-phase TLC Plates

Reversed-phased (RP) involves the use of a non-polar usually hydrocarbonaceous, stationary phase and a relatively non-polar mobile phase. Mixed RPTLC is a modification of a classical reversed phase in which a one or two carbon silane reagent was bonded to the silica gel surface to reduce silanol group activity and a liquid phase is physically coated onto the silanized silica gel (25).

Whatman KC_{18} layers consist of sorbent manufactured by bonding octadecylsilane to the surface of a special 10 u silica gel via stable Si-O-Si bonds. The layers are such as to correlate to certain C_{18} bonded (10-12% Carbon loading) HPLC high Performance reversed

phase columns. In KC_{18} precoated layers, the surface is fully covered (capped) by reacting further with a hexamethyldisilazane. KC_{18} plates contain a hard 250 μ thickness layer of octadecylsilane bonded silica gel. A typical efficiency for KC_{18} (Whatman) is 1000 theoretical plates (N) for 0.1 μg of anthracene spotted in a 1 μl volume and developed with methanol-water (90:10 v/v), where

$$N = 16 \left(\frac{\text{distance of migration}}{\text{(Width of spot)}^2}\right)^2$$

$$= 16 \left(\frac{3.4 \text{ cm}}{(0.43 \text{ cm})^2}\right)^2 = 1000$$

The principal separation mechanism on KC_{18} layers is the Van der Waals attraction between the hydrocarbon part of the sample molecules and the octadecyl groups of the layer. These forces depend in part on the carbon chain length, being approximately twice as great for C_{18} groups as for C_8 groups. Non-polar solutes have greater retention (lower R_F values) as chain length increases (26). Control of selectivity through the modification of mobile phase is the most popular approach to RPTLC.

Description and Characteristic of the Spectrofluorometer

All fluorescence measurements in this study were performed with a Farrand Spectrofluorometer, MK 2, and its

attachments. This instrument, as shown in Figures 1 and 2, is a double beam dual monochromator with an autoprocessor-1. This is a microprocessor system used in conjunction with real-time corrected spectra, differential fluorescence, ratio fluorescence, absorption and transmission (27).

The wavelength range of this instrument is 200 -700 nm standard (optional 300-800 nm) with adapters to extend the range from 400 to 900 nm for both the excitation and emission monochromators. Emission from a 150 Watt dc Xenon arc lamp comprises a good continuum throughout the ultraviolet, visible and near infrared spectral regions (200 nm to beyond 1400 nm). A magnetic xenon arc stabilizer is included to provide a magnetic field around the lamp which eliminates arc fluctuation resulting in completely stabilized emission and longer lamp life.

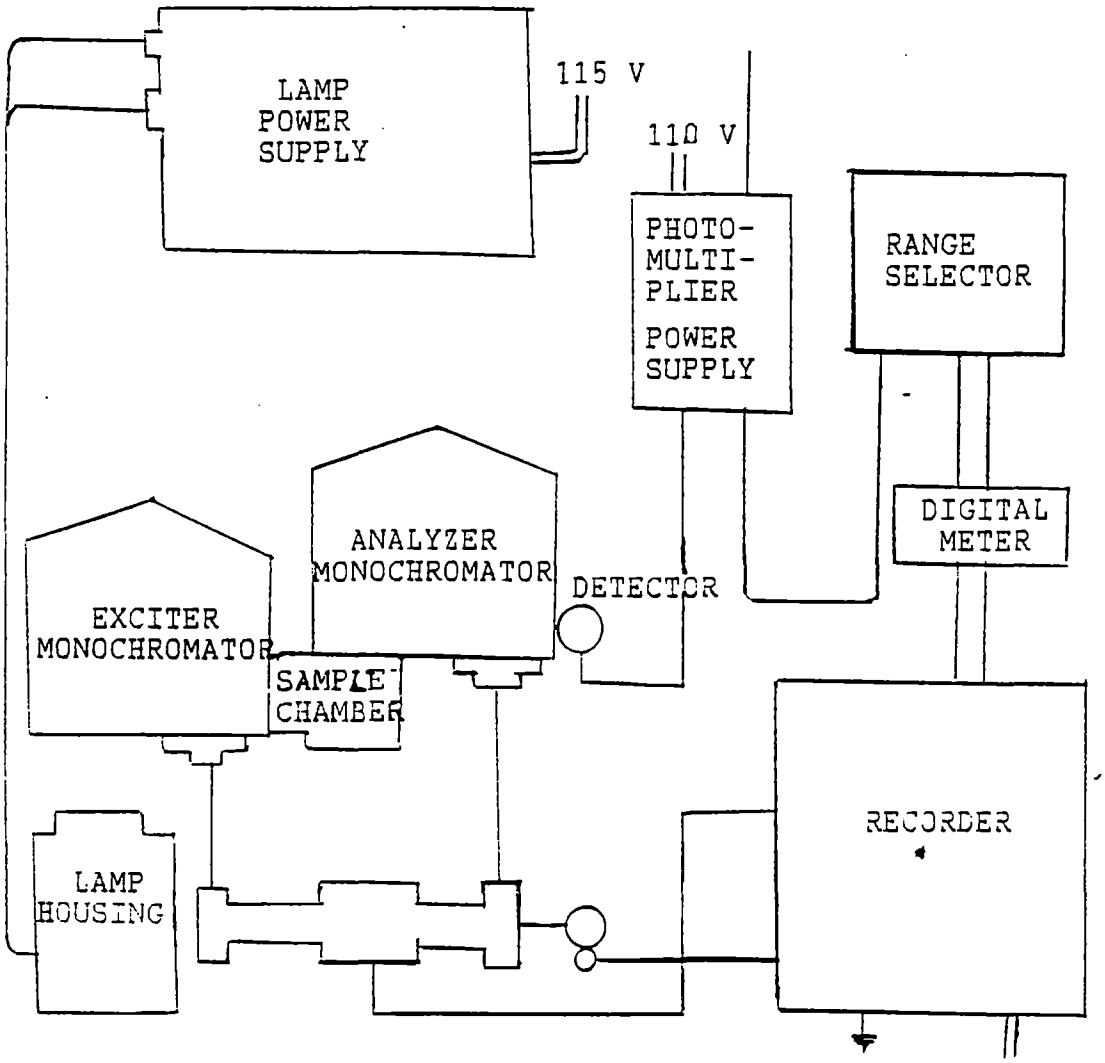
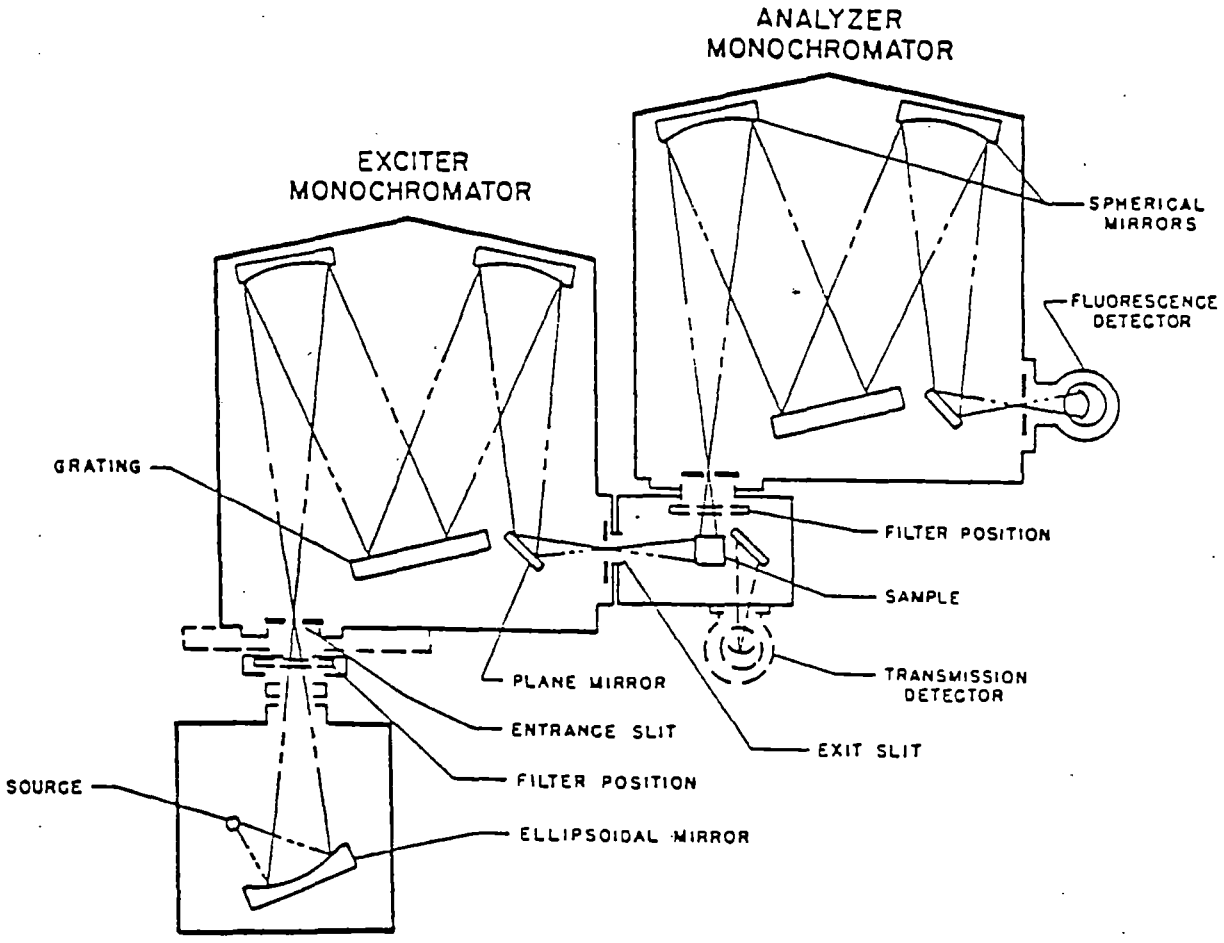


Figure 1. Block and Wiring Diagram for Spectrofluorometer



Source: Farrand Spectrofluorometer MK2, Operation and Service Manual, Farrand Optical Co. Inc. : New York, (1979).

Figure 2. Optical Schematic Diagram

The off-axis ellipsoidal mirror collects the emission from the lamp and focuses an enlarged image of the lamps arc upon the entrance slit of the exciter monochromator (See Figure 2). The source lamp and the off-axis ellipsoidal reflector are mounted in an intergrated assembly enclosed in a sealed housing. The source lamp power supply operates on 115 or 230 volts ac. External adjustments for critical focus and lateral positioning of them are incorporated. Forced-air cooling maintains a constant temperature environment.

In the monochromators, the first spherical mirror collects and collimates all radiation passing through the entrance slit. A reflection diffraction grating disperses the radiation and directs it to the second spherical mirror. This mirror focuses the dispersed spectral components upon the exit slit by way of a diagonal mirror. The radiation then centers the sample component of the phototube compartment.

The spectrofluorometer MK 2 graphing have 28,800 lines per inch on an area of 50 X 50 mm. First order dispersion is 5nm/mm. The wavelength is selected by rotation of the monochromator dials which controls the angular positions of the grating. The monochromator accuracy is better than 1 nm of the specified wavelength. Scanning speeds are 5, 10, 20, 40, 50, 100, 200 and 400

nm/min.

The specific band pass is determined by the selection of appropriate interchangeable slits for the entrance and exit positions of the monochromators. Externally interchangable metal slits have equivalant band widths of 2.5, 5 and 10 nm. Filters can be placed in front of the entrance to block unwanted radiation. A second filter position is in the sample chamber.

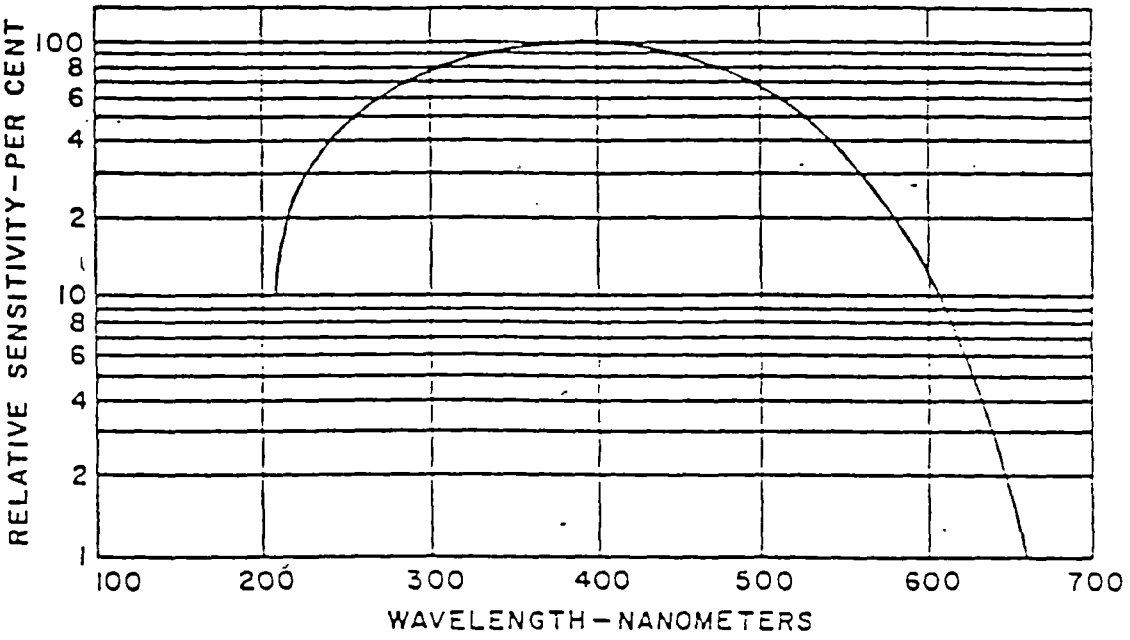
The sample chamber is completely isolated from the heat of the source lamp to assure stable sample temperature environment. There is an optional beam intensifier assembly which consists of two mirrors at right angles to each other in a holder which fits over the standard cell holder. The assembly serves two functions:

1). It reflects excitation energy, which normally passes through the cell, back into the cell;

2). Since fluorescence radiates in all directions, the second mirror reflects that portion of the emission which is radiated at 180 degree from the entrance slit of the analyzer monochromator back into the analyzer monochromator system.

The net result is a 2.5-3x increase in signal intensity.

A 1P28B photomultiplier tube is standard equipment for the Spectrofluorometer MK 2. The spectral responses range from 200 to 660 nm with peak responses at 400 nm.



Source: Farrand Spectrofluorometer MK2, Operation and Service Manual, Farrand Optical Co. Inc, : New York, (1979).

Figure 3. Spectral Characteristics of 1P28B Photomultiplier Tube

Current amplification is 5 X 10^6 with excellent signal-to-noise ratio. A typical spectral response is shown in Figure 3. The photomulitplier tube is mounted in a detachable housing. It is equipped with a knob which can be rotated manually to open and close the shutter.

The spectrofluorometer MK 2 is equipped with a digital meter. It is a 3-digit, 7-segment light emitting diode display It is also equipped with outputs for strip-chart recorders or an oscilliscope. Instrumental sensitivity of this spectrofluorometer is less than 1 part per trillion of quinine sulfate which can be determined by using a 10nm band pass in each monochromator. The signal-to-noise ratio is less than 5% of full scale at the most sensitive instrumental setting.

The Fluorescence Process

Fluorescence is one of the effects of the interaction of matter and light. Fluorometry specifically deals with one of the modes of energy dissipation that a specie undergoes once absorption has occurred. In fluorescence spectroscopy, the energy dissipation of interest is the re-emission of light which is of lower energy than the radiation which was absorbed.

Every molecule possesses a series of specifically spaced electronic energy levels (S) which are unique to

that molecule. Within each electronic level there is a series of vibrational energy sublevels (V). Under ordinary conditions the electrons in the molecule will be in the lowest vibrational sublevel of the lowest (ground) electronic energy level. In the ground state of most molecules, the electron is spin-paired with another electron and the state is called a singlet state.

Absorption occurs from the zeroth vibrational sublevel to various vibrational sublevels of an upper (excited) electronic level (S_1, see Figure 4). The actual time required for a molecule to go from one electronic state to another is 10^{-15} sec. (according to the Franck - Condon principle) which is short relative to the time required for all other electronic processes and for nuclear motion. This means that immediately after excitation, a molecule has the same geometry and is in the same environment as it was in the ground state. The molecule then undergoes a deactivation process to return to the ground state. This deactivation begins with dissipation of energy as heat by vibrational relaxation (VR) and/or intermolecular collisions (IMC) with other molecules until the electrons have reached the lowest vibrational sublevel of the lowest excited singlet states.

Once a molecule arrives at the lowest vibrational sublevel of an excited singlet state, it can do a number of things, one of which is to return to the ground state

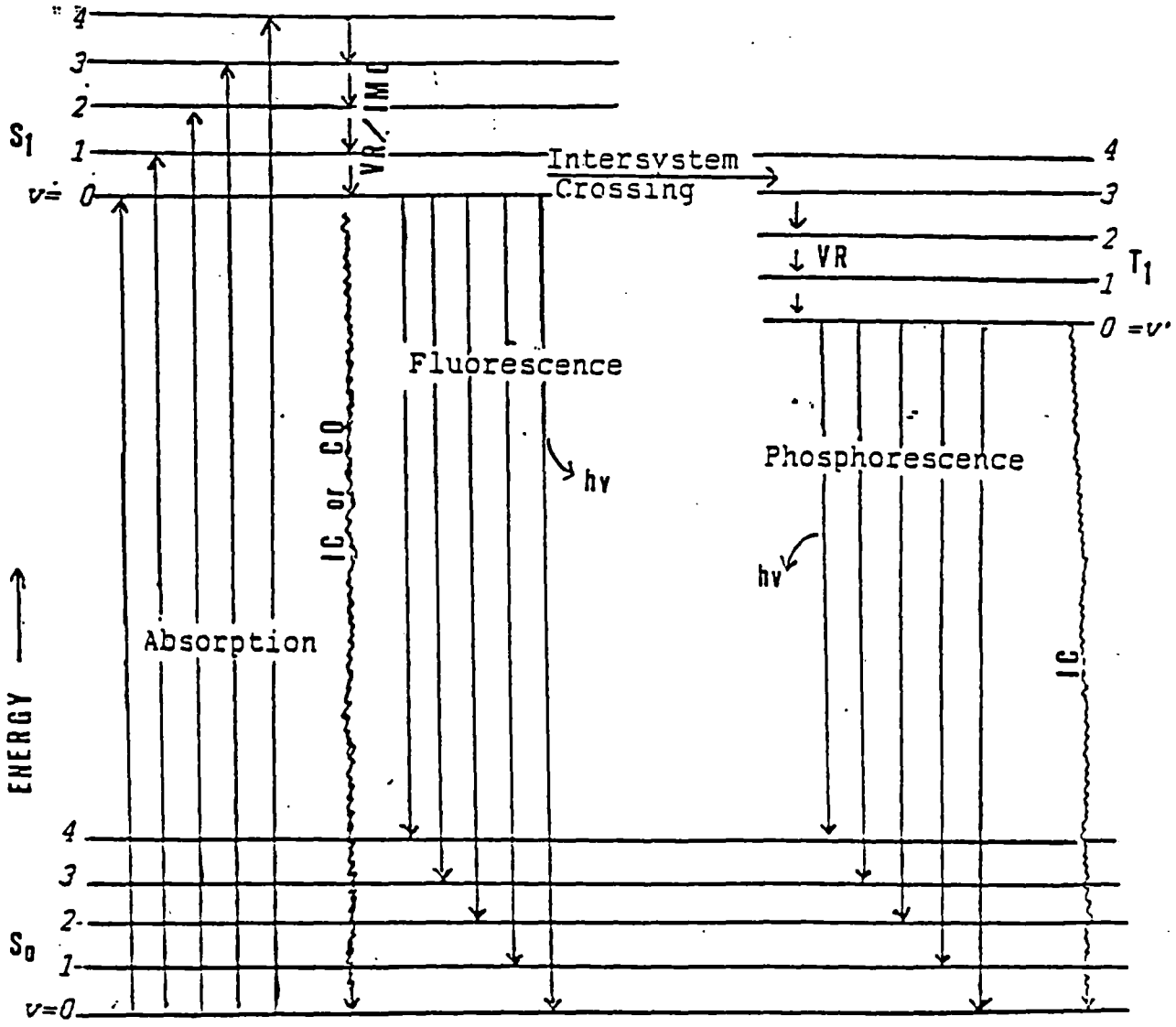


Figure 4. Schematic Energy Level Diagram Illustrating the Energy Changes Involved in Absorption, Fluorescence and Phosphorescence

See Text for Abbreviations.

by photon emission. This process is called fluorescence (F). The lifetime of an excited singlet state is approximately 10^{-9} to 10^{-7} sec and therefore the decay time of fluorescence is of the same order of magnitude. If the fluorescence is unperturbed by competing processes, the lifetime of fluorescence is the intrinsic lifetime of the excited singlet state (28)

The molecule can undergo collision with another molecule (collisional quenching, CQ) to dissipate energy and return to the ground state. The molecule in an excited state may also return to the ground state without the emission of a photon, a radiationless process in which all the excitation energy is converted to heat. The process is called internal conversion (IC). The molecule can also undergo intersystem crossing (ISC) to a vibrational sublevel of a triplet state (T_1) which involves a change in the spin of the excited electron. From the triplet state the electron can return to the ground state by phosphorescence emission (P) of a photon or by internal conversion.

It should be noted that even though a quantum of radiation is emitted in fluorescence, this quantum will be of lower energy on the average that the quantum absorbed by the molecule, due to vibrational relaxation (both after absorption and after emission). A measure of how much energy is dissipated in the fluorescence mode of

deactivation is known as the quantum yield, i.e. the ratio of number of quanta emitted to the number of quanta absorbed (29).

In summary, the process of fluorescence consists of photon absorption by a molecule to go to an excited singlet state, relaxation from higher vibrational levels of that state to its lowest vibrational level, photon emission to a vibrationally excited level of the ground state, and again relaxation of the molecule to the lowest vibrational level of the ground state.

Sensitivity and Specificity of Fluorescence

Fluorescence spectroscopy has assumed a major role in analysis, particularly in the determination of trace contaminants in the environment, industries, and body tissues. For applicable compounds, fluorescence gives high sensitivity (in the low parts per trillion) and high specificity (30).

High sensitivity results from the difference in wavelength between the exciting and fluorescence radiation. This results in a signal contrasted with essentially zero background; it is always easier to measure a small signal directly than as a small difference between two large signals as is done in absorption

spectrophotometry. High specificity results from dependence on two spectra; the excitation and the emission spectrum. Two compounds that are excited at the same wavelength are readily differentiated without the use of a chemical separation techniques. Also, a fluorescent compound in the presence of one or more non fluorescent compounds is readily analyze fluorometrically even when the compounds have overlapping absorption spectra.

Indan-1, 3-Dione Derivatives

Pindone is a water-soluble compound whose salt is know as Pivalyn. It is an anticoagulant rodenticide with delayed effects on the prothrombin level and consequent disturbances in blood coagulation, producing death by hemorrhaging. When tested in rats (single intra peritoneal injection), pindone proved to be more toxic than warfarin, but it is apparently less potent than warfarin by multiple daily doses (31).

Chlorophacinone has a toxicity rating of 5-6. Application of a solution of 5 mg in 2 mg of liquid paraffin to shaved rabbit skin produced only a slight reduction in prothrombin level. After single lethal doses in rodents, death tends to be delayed for 5-8 days and is presumably due to internal hemorrhages. Vitamin K_1 is an antidote by antagonizing the inhibition of prothrombin

synthesis, but chlorophacinone is said also to uncouple oxidative phosphorylation (31).

Diphacinone is an anticoagulant rodenticide with delayed effects on the prothrombin level and consequent disturbance in blood coagulation, resulting in death by hemorrhage. It is more potent in multiple dose experiments and especially after single dose exposure (31). Diphacinone was not extensively metabolized in rats. Less than 1% of the dose was expired as CO_2. Most of the phenyl and indandionyl rings remained intact as indicated by similar metabolite pattern with indandionyl, ^{14}C and acetyl -^{14}C labels (33).

The metabolism of diphacinone in rats involved mainly hydroxylation and conjugation reactions. The proposed hydroxylated metabolic pathways of diphacinone in rats are shown in Figure 5. Hydroxylation occurred on the phenyl and indandionyl rings (32).

Investigation of diphacinone and chlorophacinone by Bullard et al (33) as livestock systemics at their laboratory required the analysis of many blood, milk and tissue samples in which concentrations ranged from trace levels to 50 ppm. According to Hoogenboon et al (10), diphacinone could not be chromatographed successfully using a neutral or acidic eluent. The incorporation of an alkaline buffer in the eluent gave better results, but

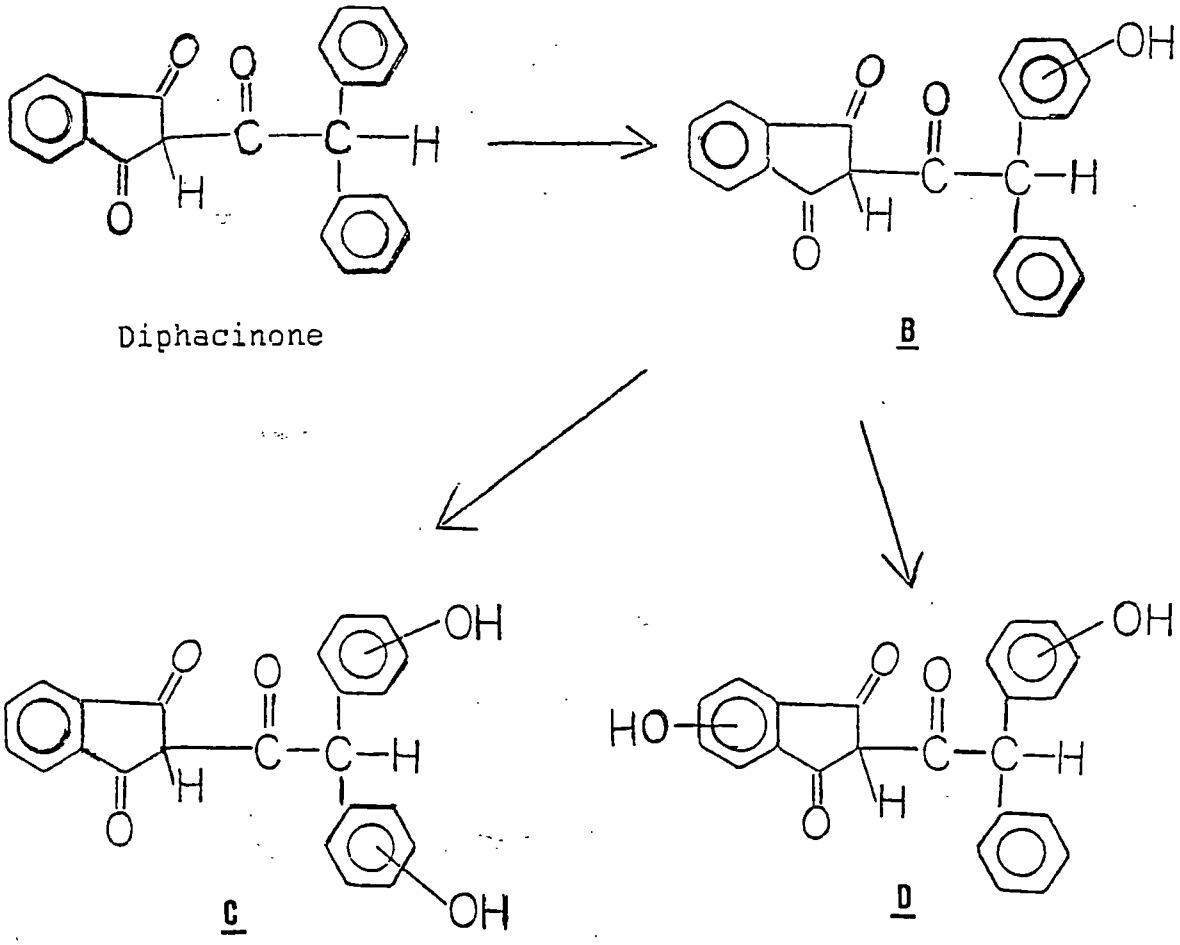


Figure 5. Proposed Metabolic Pathways of Diphacinone in Rats

the diphacinone peak was still too broad and overlapped with a small interfering peak. With the addition of an ion-paired reagent in the buffered eluent, diphacinone behaved essentially as a non-ionic species and it was efficiently separated by HPLC. This led to the addition of tetrabutylammonium dihydrogen phosphate as an ion-pair reagent for the development of the TLC plate.

Fluorescent Complexes of ß-Diketones

As seen in Figure 6, the anticoagulants studied are ß-diketones that can form enolate anions. The excitation spectrum of these complexes (i.e. complexes of ß-diketones and europium (III)) is characterized by a broad band attributed to the organic ligand and the emission is characterized by line-like emissions of the 4f levels of europium. The mechanism of the fluorescence has been described (11). This process has provided the basis of several sensitive methods for the determination of europium (13, 34) and only recently been applied to the determination of ligands (35).

The fluorescence of the europium ß-diketone complex has several positive attributes that makes it an excellent tool for the determination of the ligand. The emission of the europium is very intense due to the channeling of the

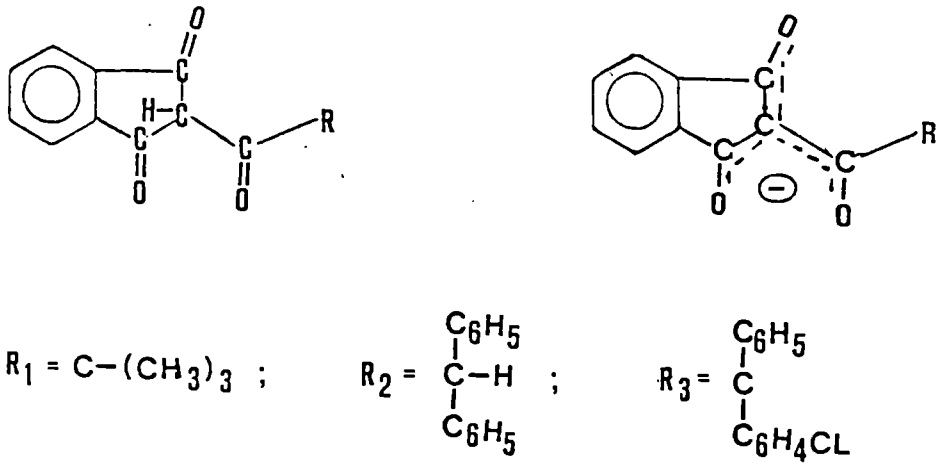
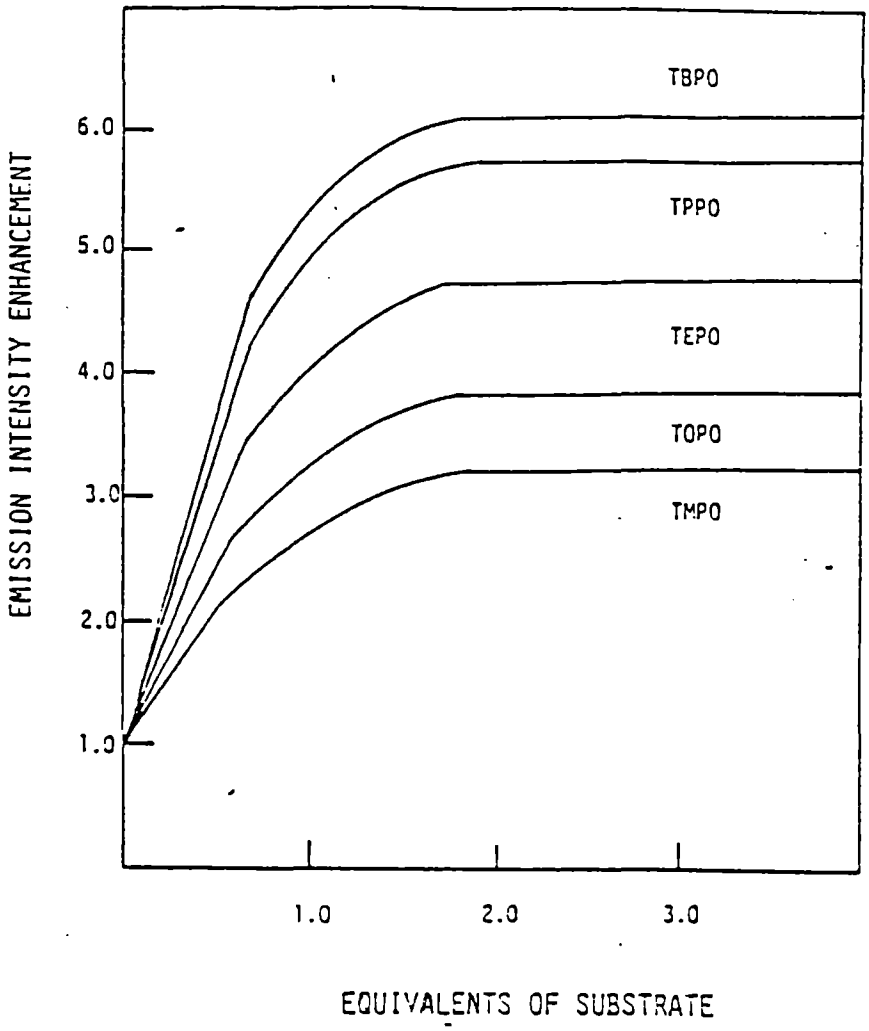


Figure 6. Structure of B-Diketones and Enolate Anions

broad band of excitation energies of the ligands into the line-like emission of the europium (35). The emission of europium is spatially removed from other emissions of which might interfere with the determination of the ligand. ß-diketones which do not under normal conditions fluoresce can be determined by fluorescence after complexation of the europium ion (35). This is desirable because fluorometric methods generally have lower detection limits than spectrophotometric methods. Addition of trioctylphosphine oxide (TOPO) and other similar compounds (synergistic agents) to solutions of europium chelates of ß-diketones produces significant increase in fluorescence emission by the chelate. (Figure 7).



Source: Benanti, S. ; Brittain, H. G. Synth. React. Met.-Org. Chem. 1983, 13, 509.

Figure 7. Luminescence Titration Curves of $Eu(TTFA)_3$ in $CHCl_3$ with Trimethyl (TMPO), Triethyl (TEPO), Tripropyl (TPPO), Tributyl (TBPO), and Trioctyl (TOPO) Phosphine Oxides. The Intensity Scales are Relative to that of $EU(TTFA)_3$ in $CHCl_3$ ($^5D_0 \rightarrow {}^7F_2$ Transition)

CHAPTER III

EXPERIMENTAL

The experimental procedures can be divided into three main categories. The first procedure was to find the R_F values between the three anticoagulant rodenticides i.e. Diphacinone, Chlorophacinone and Pindone. The next step was to find the best way to extract 0.005% Diphacinone from a commercial rat bait and to separate the extracted solution by reversed-phase TLC. The final step included the detection of the separated substance on the TLC plate and quantitation via europium sensitized fluorescence.

Preparation of Reagents

A stock solution of 0.005M ion-pair chromatograpic A (PIC A) reagent was prepared by transferring 5.0 ml of 1.0M tetrabutylammonium dihydrogen phosphate (Aldrich Chem. Co, which was used as received) into a 1000ml volumetric flask and diluted to the mark with deionized water. The pH of the solution was brought up to pH 7.5 with a 0.2M NaOH (97% Alrich reagent ACS) and measuring the pH by a Ph meter (Orion Research, Model 301). Deionized water used throughout this experimental procedures was further purified by distillation in an all

glass still with a quartz immersion heater.

The methanol + PIC A stock solution was prepared by the addition of 20 ml of the PIC A reagent and 100 ml of water to 900 ml of methanol (used as received from Fisher, certified ACS). The solution was thoroughly mixed by shaking. The water + PIC A reagent was made up by adding 20 ml of PIC A reagent to 1000 ml of water in a volumetric flask.

In the preparation of the spraying reagent, most of the solutes used such as trioctylphosphine oxide (TOPO) and europium nitrate ($Eu(NO_3)_3$) were dissolved in acetonitrile. The solvent used for fluorometric analysis was also acetonitrile. Acetonitrile was used because in fluorometric analysis the solvent is a very important variable. The choice of solvent should include the consideration that it should not absorb or fluoresce in the frequency region of interest, and it should, of course, dissolve the solute under study. In addition it is usually desirable to avoid solute-solvent pairs which exhibit pronounced hydrogen-bonding tendencies (28).

A 1.49×10^{-3}M stock solution of $Eu(NO^3)_3$ was prepared by dissolving 0.1310 g of Eu_2O_3 99.99% (used as received, Pflatz and Bauer, Stamford, CN) in 1 ml of water in a small beaker. The mixture was warmed gently on a hot plate until all the europium oxide had dissolved. It was then quantitativly transferred to a 500 ml

volumetric flask and diluted to the mark with acetonitrile (used as received, Fisher, Certified ACS).

A stock solution of 0.035 M TOPO (Aldrich, Chem. Co.) in acetonitrile, which acts as a synergistic agent (37) for the ligand was prepared by dissolving 1.37 g of the reagent (used as received) in 25ml of acetonitrile with gentle heating on a hot plate until all the reagent had dissolved. The warmed solution was quantitatively transferred to a 100 ml volumetric flask and diluted to the mark.

0.1230 g of Diphacinone (United States Pharmacopiel Convention Inc, Rockville, MD) was dissolved in 25 ml of chloroform (used as received, Fisher, certified ACS) using ultra-sound to mix the solution. The solution was quantitatively transferred to a 100 ml volumetric flask and diluted to volume with chloroform to make a stock solution of 3.88×10^{-3}M. A 10 ml aliquot of this stock solution was pipetted into a 50 ml volumetric flask and diluted to the mark with chloroform to make a standard solution of 7.76×10^{-4}M less concentrated solutions of this standards such as 1.55×10^{-4}M, $6.20\underline{4} \times 10^{-5}$M and $4.95\underline{4} \times 10^{-5}$M were made by serial dilutions of the stock solution. The stock solutions of chlorophacinone (99.35%) Chempar Chem. Co.) and Pindone (Fluka) were prepared by dissolving a weighed amount of the standards (0.0750 and 0.0864 g respectively) in 25 ml of choloroform using

ultra-sound to mix the solution.

Stock solutions of 2.00×10^{-3}M and 3.75×10^{-3}M Chlorophacinone and Pindone, respectively, were prepared in the same manner as Diphacinone above.

Procedures

The TLC plates in these experiments were developed with methanol + PIC A and water + PIC A (7:3 v/v) which was found to be the optimum ratio. Decreasing the amount of water + PIC A decreases the polarity of the mobile phase which causes a too rapid movement of the mobile phase. Increasing the amount of water + PIC A hinders the movement of the mobile phase leading to disintegration of the silanized layer of the plate.

The solvent for the mobile phase was transferred to the developing chamber. The developing chambers used in these experiments were 7 cm diameter by 9 cm tall round glass jars. The solution was throughly mixed and left undisturbed for 15 min to allow liquid-vapor equilibrium to be reached.

The 20 x 20 cm RPTLC plates were cut into 7 x 4 cm sizes with a glass cutter. Using a microsyringe, 5 ul samples of the standards were transferred quantitatively onto the 4 x 7 cm TLC plates. The samples were spotted about 0.7 cm above the edge. Each drop was allowed to dry before the next drop was added until the 5 ul volumes

not have an area too big which would decrease the number of theoretical plates.

Two samples were spotted on each plate. When the spots were dried, the plate was carefully inserted into the chamber for development to proceed. It took about 13 minutes for the mobile phase to reach about 6 cm up the TLC plate. The plates were removed from the developing chambers to be dried in an oven (120° C) for about 10 minutes.

The mobile phase was allowed to travel about 6 cm to find the difference in R_f values between Diphacinone, Chlorophacine, and Pindone and also for the separation of diphacinone from the extracted solution of the rat baits. For the linear calibration of Diphacinone, however, the mobile phase was allowed to travel about 4 cm only (it took about 7 minutes). The reason for doing this will be explained later in the procedure for fluorometric titrations.

The warmed dried plates were removed from the oven and immediately sprayed (Altech reagent sprayer) with a solution containing 20% Eu^{+3}, 10% TOPO and 1% Pyridine in acetonitrile. The sprayed plates were visualized under short wave ultra violet light (from Spectroline) and the orangish-red fluorescence areas of the complexed ligands were marked with a pencil.

Fluorometic Titration

The marked areas on the TLC plates were scraped off using the sharped end of a spatula. The areas marked were larger than the areas where the orangish-red fluorescence were actually observed to ensure that none of the standards of the separated Diphacinone from the rat baits were left on the plate.

For the standard, the mobile phase was only allowed to travel about 4 cm above the plate so that the smaller area scraped off would not introduce any more adsorbents off the plate than necessary.

The scraped residues were transferred quantitatively into a 5 ml volumetric flask. A drop of Pyridine and 5 drops of the prepared TOPO were transferred into the volumetric flask using a Pasteur pipet. In addition, 1 ml of 0.00149M $Eu(NO_3)_3$ was also added to the mixture and diluted to the mark with acetonitrile. The components of the solution were then mixed by ultasound for 30 min to form a homogeneous mixture. The homogeneous solutions were transferred to 1.5 x 12.5 cm test-tubes where they were centrifuged for 15 to 20 minutes. These solutions were slowly decanted into vials, capped and labelled so that the fluorescence of the complexed ligands could be measured. The residues from the centrifugation were discarded. The same procedures were followed for the

preparation of the blank but the Eu^{3+} ion were excluded from the mixture.

All chromatographic procedures and fluorometric titration for the standards at different concentrations and for the extracts were done in quadruplicate to ensure correct statistical results.

Extraction of Diphacinone

The commercial products containing Diphacinone were purchased at a local farm supply store. Their trade names "Durvet-Rat" and "Mouse Bait", contain 0.005% Diphacinone mixed with inert ingredients such as oats, alfalfa and ground grains.

The first extraction was done using a mechanical shaker. The unground baits (5 g) were distributed equally into four, 15 x 2 cm test tubes using water and methanol 90:10 v/v) and shaken for 1.5 h. The extract was then filtered using a 0.45 um filter paper (Millipore Filter Corp.). This method was later abandonded because, even if separation occurred we were unable to detect it under the UV light.

The preferred extraction method found for this diphacinone baits was by Soxhlet extraction. The cellulose thimble used for this experiment had measurements of 33 x 94 mm in size (Thomas Scientific, PA.). Solvents such as ether, acetone, methanol,

chloroform and ethyl acetate were tried. None showed remarkably good separation of the anticoagulant when the extracts were submitted to TLC separation. A ternary solvent mixture of acetonitrile, acetone and chloroform (2:1:1 by volume) (36) was found to give satisfactory results. This mixture was chosen for all the extractions in this study.

Approximately 30 grams of the mixed rat baits were ground to powder. 20.0 g of the ground baits were placed into the Soxhlet thimble and extracted for 10 h using the ternary solvent mixture described above.

After the extraction was complete, the volume of the extracted solution was reduced to less than 25 ml with a rotary-evaporator, transferred quantitatively into a 25 ml volumetric flask and diluted to volume with acetronitrile. If a percipitate formed during the volume reduction the solution was filtered, the percipitate was washed with acetonitrile and the filtrate and washing transferred to the 25 ml volumetric flask for dilution. The extracted sample was then separated by TLC and prepared for fluorometric titrations as described earlier.

Instrumental Settings and Fluorometric Measurements

All fluorometric measurements were done in a type 3H UV quartz fluormetric cell (NSG Precisions Cells, Inc.,

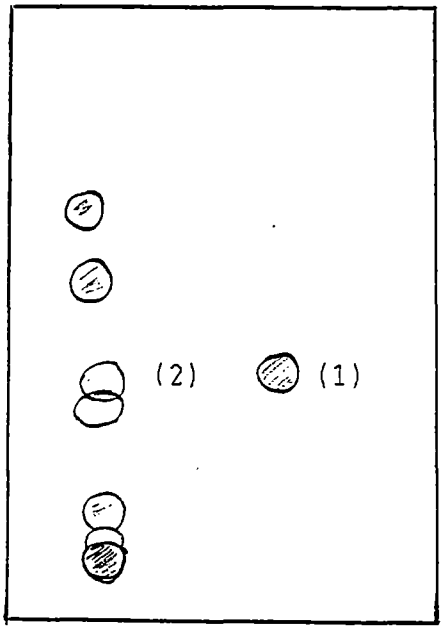
NY) with 10 mm path length and outside dimensions of 45 mm high, and 12.5 mm wide. The cells were cleaned by soaking them with methanol overnight and then air dried.

The spectrofluorometer was allowed to warm up for 15 minutes before measurements were made. With the time constant at 1.5 seconds, and the scanning speed at 20 nm/min, the optimum band widths found at the four slits for the two monochromators were 5 : 2.5 : 5 : 2.5 mm respectively (See Figure 2; from left to right). The samples was decanted into the cell, the outside walls were wiped with lens tissue before placing it in the sample holder. The fluorescence of the complex was monitored at 328 nm excitation and 613 emission. As in normal fluorescence analysis, the excitation monochromator dial was set at the desired wavelength, as the emission monochromator was used to scan over the spectral range. When the excitation spectrum was desired, the reversed procedure was followed. With the shutter closed, the samples were scanned from 550 nm to 640 nm in the emission wavelength and from 250 nm to 400 nm in the excitation wavelength. Three emission readings were taken for each of the quadruplet concentrations. Most of the readings were read from the digital meter display and recorded.

Developed TLC Plates

Figure 8 shows a thin-layer chromatogram of the

extract and standard using the proportions of the solvent systems mentioned above. During the chromatographic process an ion-pair is formed between organic-organic solute pairs, and the formation of each type is highly dependent upon the immediate environment of both ions (39). The plate was 5 x 8.7 cm and the development time was 15 min using the ascending technique. Another advantage of the RP layers is its independence from humidity; solvent demixing is also less of a problem.



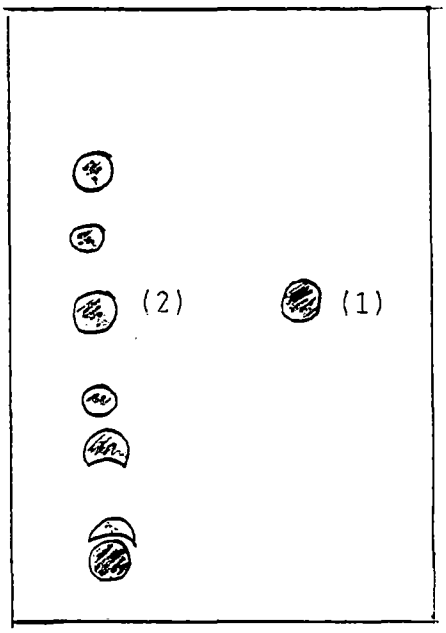
(1) Standard Diphacinone
(2) Diphacinone Separated From Extract

Mobile Phase: Methanol + PIC A and
Water + PIC A (7:3 v/v)

Plate : $KC_{18}F$ RPTLC

Figure 8. Thin-layer Chromatogram of Extract From the Rat Bait

The extract from the rat bait was also submitted to TLC separation in the reversed-phase layer using the same conditions mentioned but with exclusion of the PIC A reagent. The result (see Figure 9) showed that this mobile phase gave better separation without the use of PIC A reagent. The good separation is due to the Van der Waals attraction between diphacinone and the octadecyl groups of the KC_{18} layers of the TLC plates. The addition of PIC A reagent, makes the mobile phase bulkier, hence hinders the separation of the extract.



(1) Standard Diphacinone
(2) Diphacinone Separated From Extract

Mobile Phase: Methanol + Water (7:3 v/v)

Plate : $KC_{18}F$ RPTLC

Figure 9. Thin-layer Chromatogram of Extract From the Rat Bait

R_F Values Between Anticoagulants

R_F value of a compound is given by the fraction of the distance of the solute to the solvent. Kirchner, et al, (40) in 1951 first showed that with careful control of conditions (standardization), the reproducibility of R_F values could be held within uncertainties of ±0.05. Two of the more important factors for control of the R_F values are uniform thickness of the layer of adsorbent and moisture in the adsorbent. These factors are of less importance nowadays (25).

The RPTLC used in this study came in a uniform layer of adsorbent, hence reproducibile R_F values can be obtained easily. The standard deviations are taken from three developments (See Table IV). The values are small indicating good reproducibility of the R_F values. R_F values are highest for Pindone followed by Diphacinone and Chlorophacinone. The difference in formula weight of these compounds (230.2, 340.4 and 374.8 g mol^{-1}, respectively) could be the factor for these differences in R_F values i.e. as a rule of thumb the smaller the formula weight the faster the molecule is eluted.

The reason for finding the R_F values between these three anticoagulants is that, these three compounds all having a ß-diketone structures gave maximum emission at 613 nm and 328 excitation via europium-sensitized

TABLE IV

R_F VALUES FOR CHLOROPHACINONE (CPCN), DIPHACINONE (DPCN) AND PINDONE (PDN)

	CPCN	DPCN	PDN
No. of mmoles	8.0×10^{-6}	4.23×10^{-6}	1.13×10^{-6}
1st R_F value	0.465	0.657	0.808
2nd R_F value	0.453	0.648	0.792
3rd R_F value	0.471	0.653	0.827
average R_F value	0.463	0.653	0.809
standard deviations	0.009	0.005	0.018
Color of Europium Complex	Orangish-red	Orangish-red	dark spot

Mobile-Phase : Methanol + PIC A and Water + PIC A (7:3 v/v)

Plates : $KC_{18}F$ RPTLC Plates

Spraying Agent : 20% $Europium^{3+}$, 10% TOPO, 1% Pyridine } in Acetonitrile

fluorescence. The difference in R_F values between these compounds is qualitative information to distinguish one compound from another using a simple sensitive technique.

Detection and Formation of Europium Complex

The $KC_{18}F$ plates used in this study were precoated with silica gel F_{254} (Merck) containing zinc orthosilicate which gives a green fluorescence at 254 nm i.e. at the wavelength of shortwave UV light. The compounds that are separated by this TLC which absorb light in the ultraviolet region but without emission in the visible region were detected as dark spots at this wavelength.

The sensitivity of this method depends on the molar absorptivities and the coincidence between the excitation spectrum of the luminophore and the absorption spectrum of the sample substance (41). Although the luminophores used in TLC are very efficient in detecting separated compounds, the selectivity they offer is poor because the substance appears as colorless spots.

According to spectroscopic and x-ray crystallographic evidence, Diphacinone, Pindone and Chlorophcinone having ß-diketone structures exits predominately in the enol form (42,43) and they are known to from salts with alkali metal ions (5). Latimer (36) found the possibility

of applying europium-sensitized fluorescence to the determination of these compounds. With this information in hand, we investigate the possiblity of detecting these compounds by spraying the above mixture on the developed plate. An orangish-red fluorescence could be observed where the anticoagulants were and dark spots were seen for other separated components.

As seen in table IV the dark spot seen for Pindone may be due to the compounds present in the adsorbent layer of the KC_{18} RPTLC plate which quench the intensity of the fluorescence formed. The orangish-red fluorescence color of the Pindone complex can be observed however, on a normal TLC plate such as one having calcium sulfate as binder.

For the europium-ligand complex to be formed, the ß-diketone must be in the enolate anion form. The 1% pyridine added acts as a base in order to remove the acid proton of the ligands and form the enolate anion necessary for complexation of the europium. Latimer (36) reports that with pyridine above 1% the fluorescence decreases.

Addition of TOPO insulate the fluorophore from the solvent, reducing the solute-solvent interaction and

increasing the intensity of the fluorescence. TOPO presumably replaces water molecules coordinated to the europium ion, reducing solute-solvent interaction and collisional deactivation.

Spectrums of the Complexed and Uncomplexed Ligands

The natural fluorescence of diphacinone is not analytically useful due to concentration quenching and inner filter effects. Figure 10 shows the emission spectra of the complexed and uncomplexed ligands at 8.461 x 10^{-7}M. The uncomplexed ligand had the same ingredients as the complexed ligand with the exception of Eu^{3+}. Instrumental setting were the same for both spectra. The emission spectrum of the complexed ligand shows a characteristic line-like emission of the 4f electronic levels of the europium (III) ion.

Figure 11 shows the excitation spectrum of the complexed ligand from 270 nm to 400 nm. The shape of the excitation band is broad and characteristic of an organic ligand as expected.

The fluorescence of the ligands should be measured directly after preparation since the intensity of the fluorescence decreased after several days. Figure 12 shows the difference in emission spectra of the complexed ligand freshly measured, and on the 7th day after its preparation.

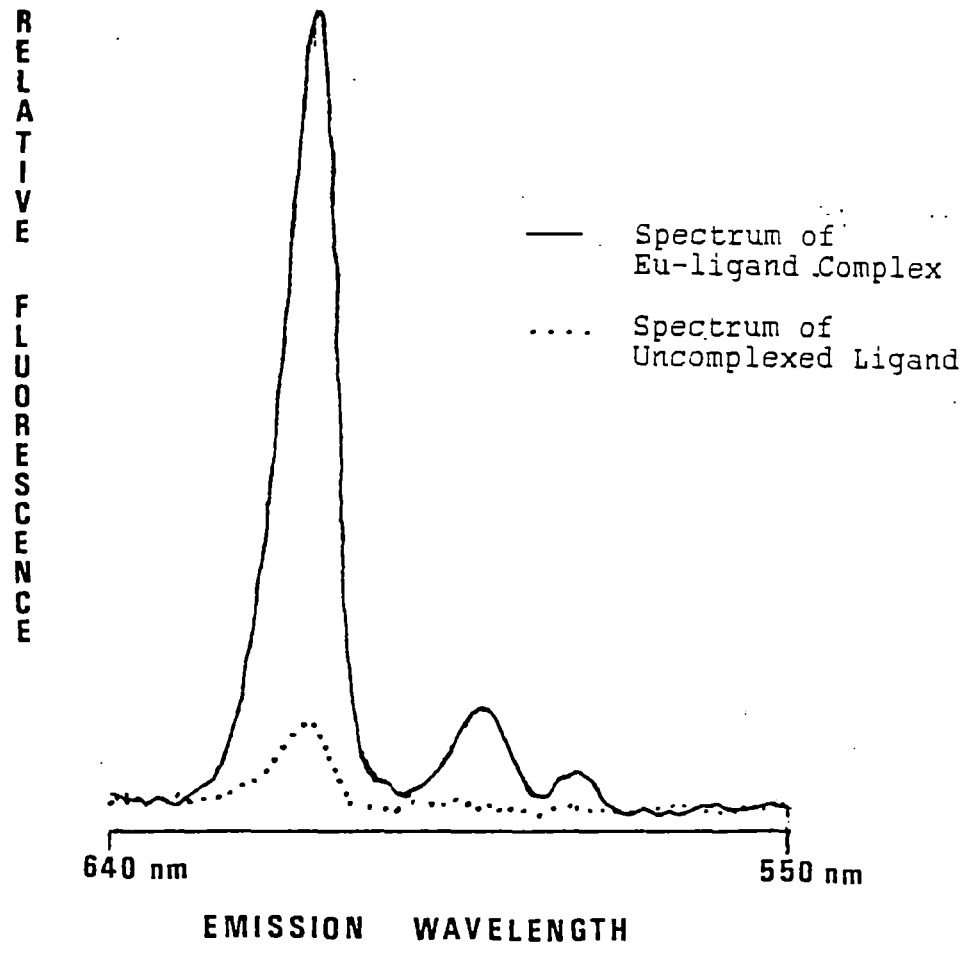


Figure 10.. Emission Spectra of the Complexed and Uncomplexed Ligands at 8.461 X 10^{-7}M

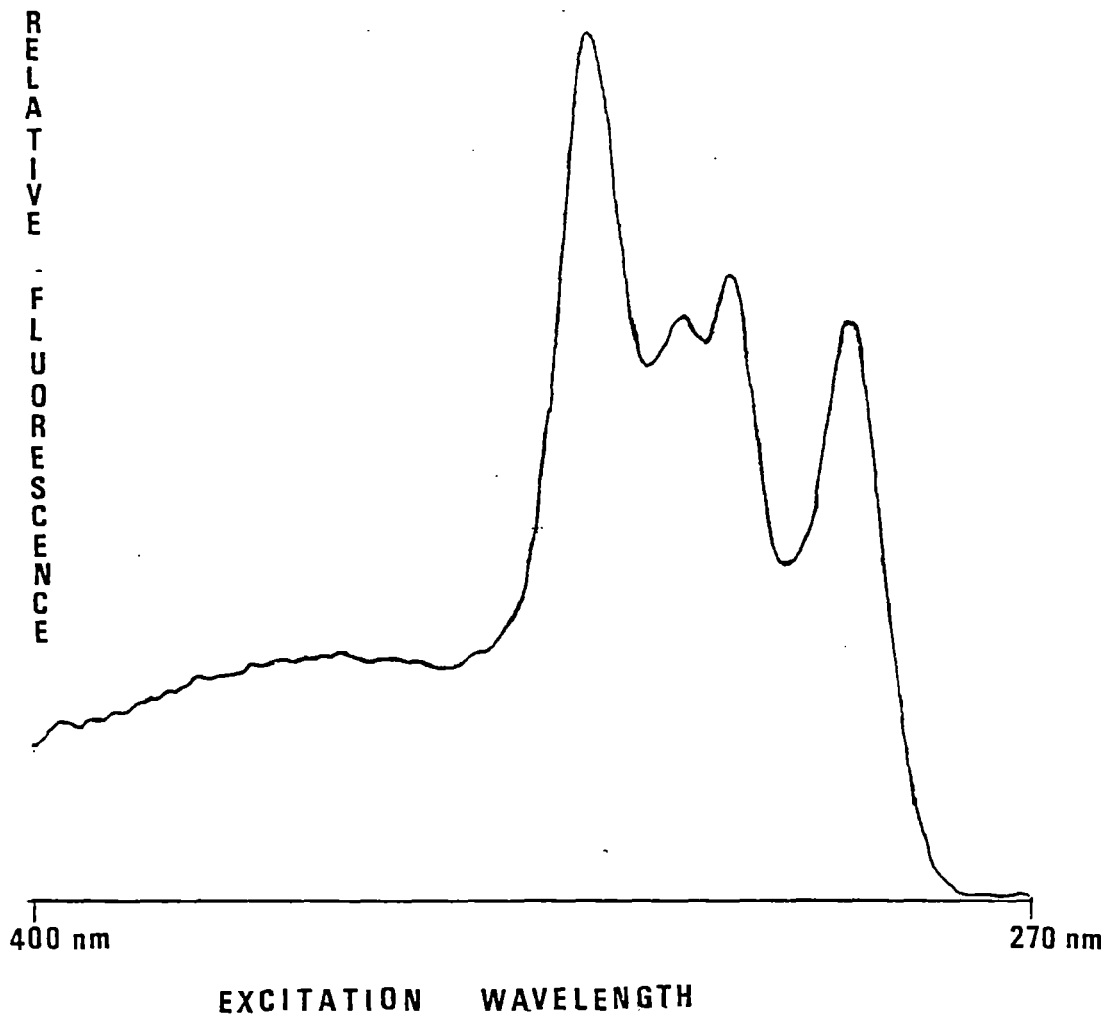


Figure 11. Excitation Spectrum of Complexed Ligands
Emission Wavelength 613 nm

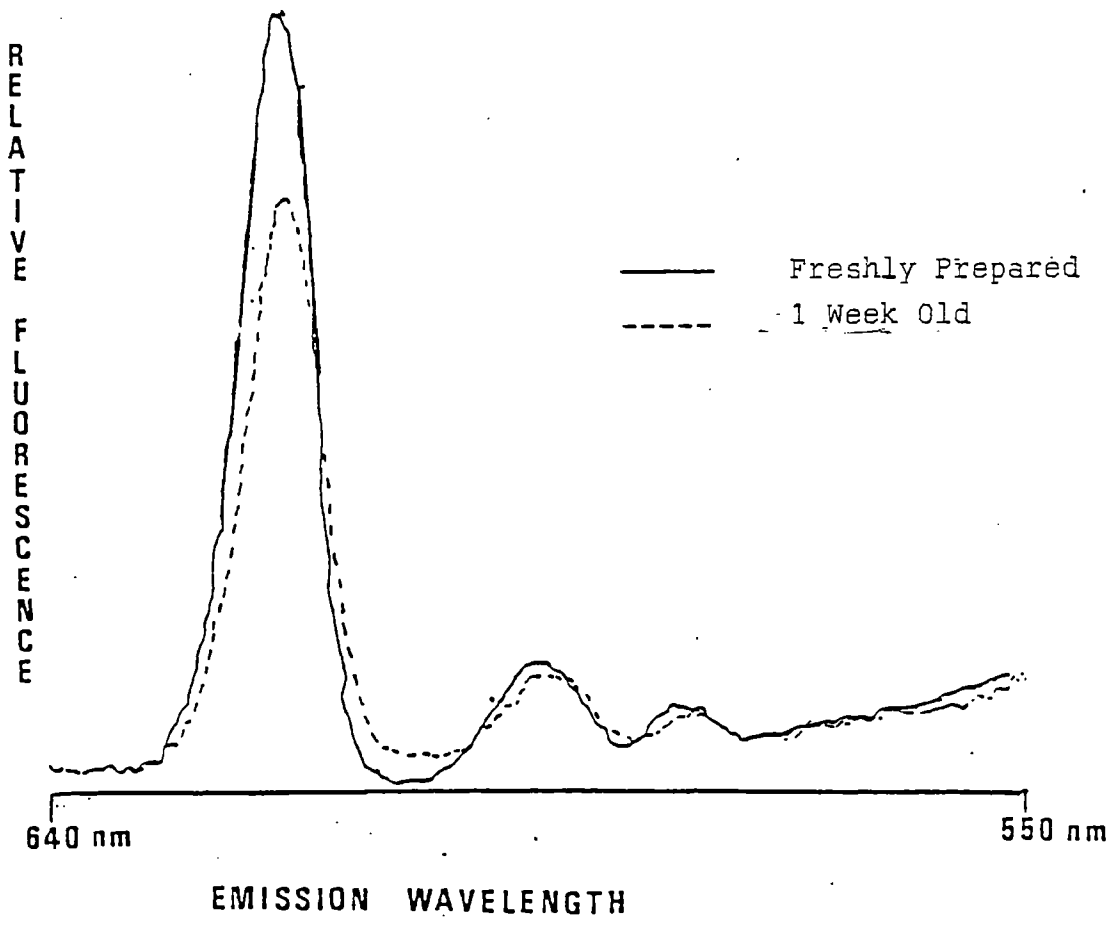


Figure 12. Difference in Emission Spectra Between Freshly Prepared Sample and a One Week Old Sample

CHAPTER IV

RESULTS AND DISCUSSIONS

Emission of Standards

Fluorescence readings were taken for the standards as summarized in Table V. Even though quadruplicate samples were prepared for one concentration, the emission readings were the average of three readings with close emission values. Some of the standard deviation values were high due to inner filter effects. This was caused by small particles that got into the cuvet from the adsorbent layer of the TLC plate and/or the high concentrations of the standard. 4.96×10^{-9} M was the lowest concentration that was able to be detected with confidence. Lower concentration gave fluctuating values from the emission readings.

Linear Regression Curve

The linear regression curve of the relative fluorescence vs. concentrations of the standards were plotted in Figure 13 (calculated and graphed by a Hewlett Packard 85 desktop computer). The emission readings of the standards yielded good linearity as indicated by a correlation coefficient of $r = 0.984$

TABLE V

FLUORESCENCE READINGS OF THE STANDARDS AT DIFFERENT CONCENTRATIONS

Concentration (M)	Relative Emission (X)*	Y
3.88×10^{-6}	13.97 (6.04)	13.63
1.55×10^{-6}	4.65 (1.19)	5.74
7.76×10^{-7}	3.27 (1.89)	3.11
3.10×10^{-7}	2.22 (0.53)	1.53
2.82×10^{-7}	1.40 (0.69)	1.44
6.20×10^{-8}	1.38 (0.47)	0.69
1.24×10^{-8}	0.24 (0.11)	0.53
9.93×10^{-9}	0.43 (0.07)	0.52
4.96×10^{-9}	0.13 (0.03)	0.41

(X)* Standard Deviation 3 Emission Reading

Y Estimated Emission Readings from Linear Plot

r = Correlation Coefficient
= 0.984

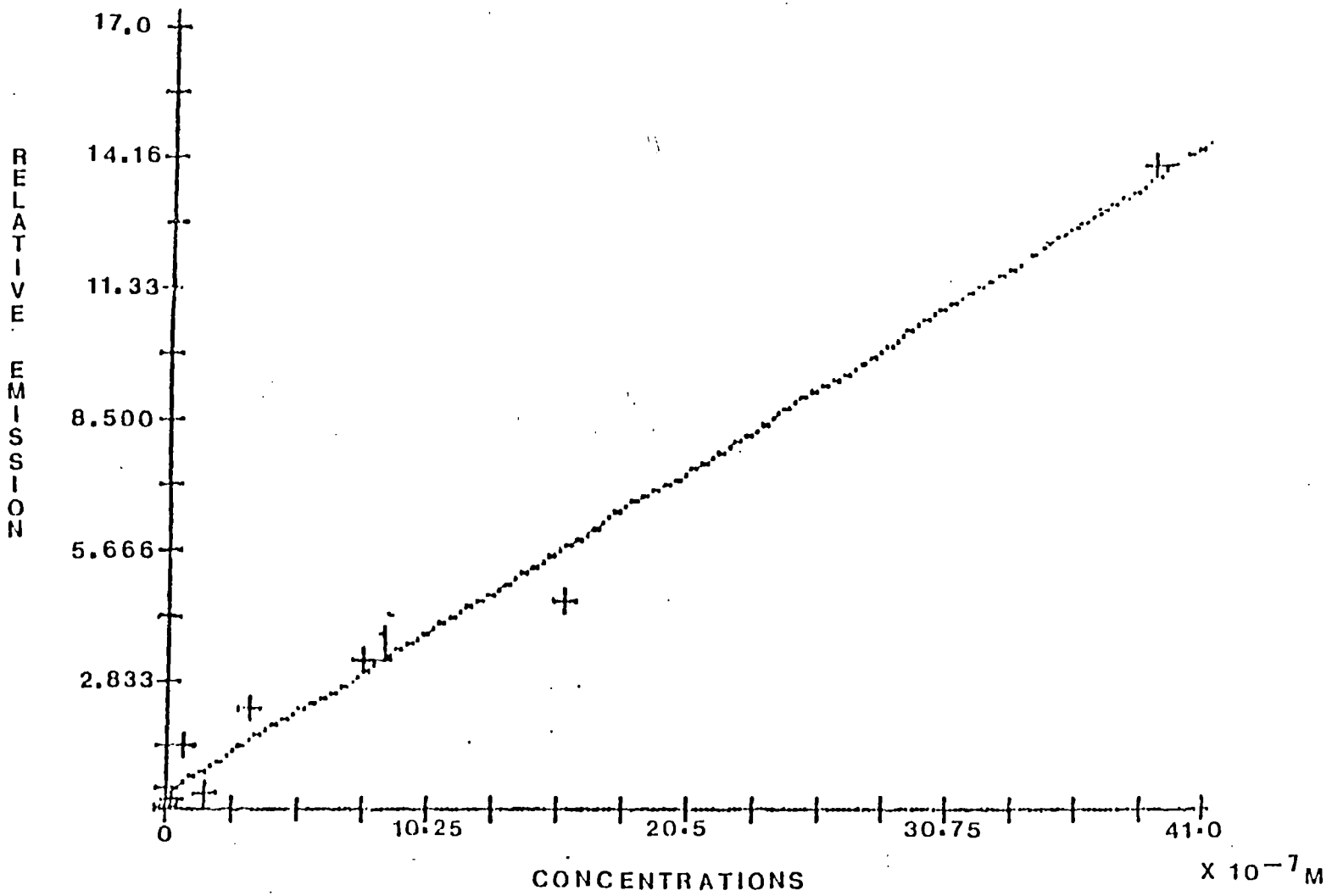


Figure 13. Linear Regression Curve of Standard at Different Concentrations

Quantitative Analysis of Extracts

As shown in Table VI, the percent Diphacinone calculated from the linear regression curve range from 0.0027% to 0.0039%. As for the standards, the emission readings here are the average of 3 similarly prepared concentrations of the extracts from 4 different extractions.

TABLE VI

PERCENT DIPHACINONE FROM EXTRACTS CALCULATED FROM LINEAR PLOT

Relative Emission ($\acute{X}$)*	Concentration (M)	% DPCN
0.42 (0.014)	1.58×10^{-6}	0.0027
0.46 (0.058)	1.75×10^{-6}	0.0030
1.02 (0.466)	2.08×10^{-6}	0.0035
1.28 (0.795)	2.30×10^{-6}	0.0039

(X)* Standard Deviation Taken from 3 Emission Readings

% DPCN Percentage of Diphacinone

SUMMARY

The focus of this study was to quantitate separated compounds obtained from TLC plates. Since the compounds under study have a ß-diketone structure and can be made to fluoresce, spectrofluorometry was chosen as the method for quantification. The overall method yielded about 78% of Diphacinone from the value reported on the packages by manufacturers. This is quite acceptable since the separated component was scraped off the plate, diluted, homogenized with ultrasound, centrifuged and then analyzed by fluorescence. If in situ fluorometry could be performed, it may be assumed that a higher percentage yield could be obtained.

In addition, if future work is to be done on these ß-diketones, it is suggested that all measurements and readings be done directly after sample preparation since decomposition takes place within a few days after they were prepared. This was indicated by the blue fluorescence observed on the 7th day of measurement and the lower emission readings.

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VITA

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Master of Science

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