Liquid-liquid extraction with long wavelength fluorescence detection

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LIQUID-LIQUID EXTRACTION WITH LONG WAVELENGTH FLUORESCENCE DETECTION.

By

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B.Sc., M.Sc.

A Doctoral Thesis Submitted in partial fulfilment of the requirements for the award of

DOCTOR OF PHILOSOPHY

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Loughborough University of Technology

1994

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I would also like to thank Rashmi for his financial support, Chub, Dave and Mark for a memorable time in the lab, and the coffee club for a memorable time outside of the lab.
AIM OF THE PROJECT

The aim of the project is to investigate the possibility of using long wavelength fluorophores for the detection of named drugs using on-line liquid-liquid extraction.

The research will be carried in the following stages:

1. Design of a phase separator to be used in a flow extraction manifold, to carry out effective liquid-liquid separation;

2. Investigation of a number of long wavelength fluorophores in an attempt to establish their uses as a means of detection of named drugs, after manual liquid-liquid extraction initially and if successful using on-line extraction;

3. The study of the mechanism occurring, i.e., is it ion pairing?

4. Whether the extraction process is buffer pH or ion dependent.

5. Could the method be used for the analysis of drugs present in human serum?

6. If time permits, to investigate alternatives for chloroform as an extracting solvent.
ABSTRACT

Long wavelength fluorophores, methylene blue, Rhodamine 800, 3,3'-diethyloxacarbocyanine iodide (DODC) and 1,1',3,3',3',3'-hexamethylindotricarbocyanine perchlorate (HIDTCP) were investigated as probes to quantify drugs after liquid-liquid extraction. Fluorescence measurements in the long wavelength region (550-1000nm) are a recent development in photoluminescence spectroscopy and offer many advantages compared with conventional measurements which are made in the ultraviolet and visible spectral regions. These include, reduced background fluorescence in the presence of biological materials; reduced scattering; decreased photodecomposition and the availability of inexpensive, solid state optical components which operate in the long wavelength spectral region.

On-line liquid-liquid extraction was carried out using a phase separator, with an efficiency of separation of 96%, designed "in-house". This technique offers the advantages of low sample consumption; rapid rates of analysis and minimisation of operator contact with the extracting solvent when compared with manual extraction.

The proposed extraction manifold used was able to determine flufenamic acid, phenylbutazone and warfarin within their therapeutic ranges. Studies also revealed that the process was buffer pH and ion dependent. Ion pairs as well as differing ratios of fluorophore, depending upon the fluorophore, were found to be extracted in the presence of named drugs.

The technique was found to be suitable for the detection of the drugs when present in human serum within their therapeutic ranges.

Finally, an investigation into an alternative to chloroform (density = 1.47g/ml) as the extracting solvent using dimethylmaleonate (density = 1.154g/ml) and diethylacetooacetate (density = 1.025g/ml) proved unsuccessful.
PRESENTATION OF WORK


May 1993: 5th International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences (Poster). University of Ghent, Belgium.


PUBLICATIONS


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CHAPTER ONE

ON-LINE LIQUID-LIQUID EXTRACTION
1.1 Introduction.

Despite great improvements in the selectivity and sensitivity of modern analytical instrumentation, the classical pre-concentration and separation techniques, such as liquid-liquid extraction, ion exchange chromatography and precipitation etc, are still widely used to improve analytical measurements. Liquid-liquid extraction is one of the classical methods most often used, for sample pretreatment preceding analytical measurement giving improved selectivity and sensitivity in a vast number of determinations.

An analyte can be separated from an interfering matrix, or interfering matrix components can be removed from the sample so as to improve the selectivity of the analyte determination. In addition an analyte may be concentrated by extraction from a large volume of an aqueous sample in a comparatively small volume of an organic phase, thus improving limits of detection.\(^4\)

Manual liquid-liquid extraction procedures are normally very tedious, using large quantities of solvents and chemicals, and are liable to contamination from the atmosphere and chemical glassware, furthermore, the traditional liquid-liquid extraction process requires handling of hazardous and/or toxic organic solvents. The treatment of a larger number of samples and the introduction of human error during each extraction must also be considered.

The result of these factors is that the preparation of samples, especially if a complex matrix is involved, is usually the time-limiting step for the determination. Therefore, a need arises for easier preparation of samples, whether it be mechanized or automated, by either direct or indirect coupling of a liquid-liquid extraction manifold to the detection system, enabling the disadvantages mentioned previously to be alleviated.

The development of automated liquid-liquid extraction is probably due to the widespread use and importance of the process. One of the most practical ways to shorten the duration of this procedure has been the introduction of "on-
liquid-liquid extraction systems applying the principles of continuous flow analysis. Logical steps to automate the technique were centrifugal and air-segmented flow analyzers. However, with the introduction of suitable equipment, flow injection analysis has also been used to modify this separation technique. As liquid-liquid extraction in flow injection analysis is fast, with little interference from emulsions, and the absence of air bubbles, flow injection analysis facilitates separation of the two immiscible phases used in the extraction process.

Liquid-liquid extraction has been semi-automated by several workers, principally using air segmented flow systems. The earlier research of the viability of liquid-liquid extraction in flow injection analysis was carried out by Karlberg and Bergamin and their co-workers in 1978. Since this time a vast number of analytical procedures have been presented, in combination with many different types of detection systems. Currently, approximately 30% of all pre-concentration methods in flow injection analysis employ liquid-liquid extraction procedures. The advantages and difficulties associated with this methodology, including some analytical applications have been discussed in several publications, and a number of comprehensive reviews clear evidence for the methodology having been shown to be satisfactory.

1.2 Basics of liquid-liquid extraction-flow injection analysis.

Liquid-liquid extraction is where defined volumes of the immiscible organic and aqueous phases must first be brought into intensive contact with each other to allow the extraction to take place, and finally physically separated from each other after the extraction in order to make the chemical separation meaningful.

In conventional flow injection analysis manifolds (Fig. 1.1), aqueous sample solutions are introduced either continuously or in defined volumes into an uninterrupted aqueous stream. This stream acts as both a carrier and reagent stream in the simplest single-line manifold.
The aqueous sample solution can also be joined and mixed with another separate aqueous stream containing an organic reagent (Fig. 1.2). This method, allows chemical reactions as well as solution mixing to occur in a reaction-and-mixing coil before entering a phase separator.

The resultant, theoretically pulse-free, aqueous stream of an extractable component is then segmented with an organic immiscible solvent stream at the segmenter mixing point, where near reproducible "plugs" of one phase in the other are formed. The reproducibility of these "plugs" is determined by a combination of gravity, density, interfacial and hydrodynamic forces. The geometry of the inner capillary system of the phase separator and the quality of the surface also contribute to the plugs.
The "plug" moves into the outflow channel after formation and tends to minimize the interfacial area with the other phase and to maximize the contact surface area with the wall material of the outflow tubing, thereby wetting it. This process results in the formation of distinct, more or less, regular segments of both phases in a single stream which then enters the extraction coil.

Depending on the material of the outflow channel and the material of the extraction capillary coil, the solvent that has the greater affinity for the tubing material coats the tubing walls with a very thin film. With glass coils the walls are wetted by the aqueous phase, while the organic phase circulates as elongated bubbles, whereas with PTFE coils the converse is true.

The extraction procedure takes place mainly in the extraction coil, and to a lesser degree in the solvent segmenter and phase separator. During extraction, the extractable sample components go from a relatively uniform aqueous solution of higher analyte concentration into the segments of the immiscible organic phase concentration through the segment interface. The interfacial area available for the extraction consists of menisci between the two phases and a film surrounding the segments. An analyte diffuses to the interface between the two phases, and extraction equilibrium is attained.

Obviously, the degree of extraction is dependent upon the residence time of the analyte in the extraction coil, which in turn is a function of the coil length and flow rate. The efficiency of extraction in the reaction coil is usually high and is normally completed within a few seconds. The contribution of the flow rate and other manifold parameters (e.g., transmission tubing diameter, lengths of coil, injection loop volume), on the extraction efficiency is dependent upon the mass transport process and kinetics of the extraction process taking place. The rate of extraction increases with decreasing segment size and decreasing inner diameter of the extraction tube, i.e., the narrower the extraction tube, the smaller or shorter the segments and thus enhanced
extraction. The choice of material used for the extraction coil indirectly affects the extraction efficiency; there is a high efficiency for transporting a determinant from the aqueous to the organic phase for PTFE coils, whereas it is very low for glass coils. However, the efficiency of determinant transfer from the organic to aqueous phase is higher for glass coils than for PTFE coils.

The segments of the aqueous and organic phases are next separated into individual streams by the phase separator. Once the analyte has been extracted into the receiving phase, determination takes place via a flow through detection system. The signals are treated in the normal way, i.e., analyte concentration is calculated from peak height, peak width, or peak area. In essence, the other separated phase is directed to waste through a restrictor coil. The restrictor coil also controls the efficiency of separation of the phase separator.

1.3 Theoretical aspects.

1.3.1 Segmentation.

Segmentation of the two immiscible phases is extremely important to the information attained using liquid-liquid extraction-flow injection analysis. The entire segmentation process can disadvantage sample dispersion, extraction rate, and phase segmentation\(^\text{20}\). Two different processes govern segmentation. Initially, droplets or plugs of one phase may be produced in a continuous flow of the other immiscible phase. This process can take place either at a junction of a multi-channel inlet capillary system, at the end of a single inlet tubing capillary or in a small mixing chamber in the segmenter. The mechanism also controls the segmentation in a mixing chamber constructed of hydrophilic material, e.g., a glass T-piece or commercially available A8-T\(^\text{™}\) (Technicon) fitting at moderate flow rates. The other process governing segmentation is the "ripple" process, this is a consequence of the destruction of the thick layer of one of the solvents, which is formed at high pumping rates on
the wall of the outlet tubing or on the walls of the mixing chamber of the segmenter. This mechanism is apparent when the walls of the mixing chamber are made from lipophilic material, e.g., fluoropolymer, or at very high flow rate ratios. In addition, the walls of the mixing chamber made of hydrophilic material may become covered with a layer of lipophilic impurities, thereby altering their hydrophilic nature.

Two variables that need to be examined in relation to the segmentation process are segment size and segmentation reproducibility. Although the size of the segment may not affect the efficiency of extraction for a rapid extraction or the introduction of large sample volumes into the system, it could in theory affect the efficiency of slower manifolds. The maximum segment size is dependent upon the interfacial tension of the organic and aqueous phase, both between each other and between each phase and the tubing material, so that the segment size decreases with decreasing values of the interfacial tension between the organic and aqueous phases.

The size of the contact area between the phases is controlled by the length of the segment in the extraction coil. A non-uniform pattern of segmentation causes losses of the solvent by the wetting process as a result of varying film thickness. Extreme coalescence of segments of differing size results from differences in linear velocity of the segment having different geometries, resulting from differences in viscous drag of the wetting phase. Consequently, the segmentation pattern must be controllable and constant throughout the analytical process. Therefore, during the optimization studies for the manifold, it is important to take into account peak (sample zone) broadening, kinetic efficiency and total extraction yield.
1.3.2 Film formation.

The high efficiency of extraction in the extraction coil is explained by the process of liquid-liquid extraction-flow injection analysis in narrow capillary tubes, which is in most part as a result of the formation of a very thin film of one solvent on the inner wall of the capillary system\textsuperscript{20,24-26}, surrounding segments of the other phase. This can be clearly observed for glass and thin wall fluoropolymer tubing and indirectly for steel.

When a plug of a precisely defined volume of one immiscible solvent is introduced into a continuously flowing stream of the other solvent, the original cylindrical shape of the solvent plug inside the loop injector changes, to a spherical, an ellipsoidal, or a deformed tubular shaped segment, inside the reaction tubes the shape formed depends essentially on the ratio between the segment volume and the inner radius of the tube, and less importantly on the interfacial tension of the solvents, the surface tension of the solvents, the tubing material, the total flow rate and other parameters.

![Fig. 1.3: The shape of the segments of aqueous \(\square\) and organic \(\blacklozenge\) phases in glass (A to C) or fluoropolymer (D to F) tubing inside a sample loop of the injector (A,D) and at zero (B,E) and very high (C,F) total flow rates.](image)

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Depending on the material of the extraction capillary coil, the solvent displaying the greater affinity for the tubing material will cover its inner walls with a very thin film of near still nature. The film of organic solvent will surround the deformed spherical, ellipsoidal, or tubular aqueous segments in fluoropolymer tubing whereas the organic phase segments will be surrounded by an aqueous film in glass or metal tubing. The need for film formation is due to the minimization of the interfacial energy at the solid/liquid interface, which is dependent on the relative sizes of the surface tension of the inner wall surface of the tubing to the liquids (wetting ability) and the interfacial tension of the liquids. The thickness of the film is related to the nature of the film-forming phase in such a way that increased viscosity and/or reduced interfacial tension results in a thicker film. The thickness of the film depends on the flow rate, flow rate ratio, the alternating frequency and segment length ratio.

It may, in practice be expressed in the form

\[ d_f = kr_o \left( \frac{u \cdot \eta}{\gamma_{oa}} \right)^a \]

where \( r_o \) is the inner radius of the tubing, \( u \) is the linear velocity of the flowing stream (in \( \text{cm/s} \)); \( \eta \) and \( \gamma_{oa} \) are viscosity (in poise) and surface tension (in \( \text{dyn cm}^{-1} \)); \( a \) and \( k \) are constant terms which are usually equal to 0.5 or to 0.67. The effect of the properties of the film-forming phase depend on the \((\eta/\gamma_{oa})^a\) factor (equation 1.1).

Another parameter affecting the formation of the wetting film are the hydrodynamic forces connected with mass transport due to the velocity distribution of the laminar flow across the tube profile, as the segment of non-wetting solvent forms a compressible "ball" flowing through the stream of the other solvent. As well as other parameters affecting the shapes of the segment, the average value of the film thickness of one liquid behind a single plug of another (immiscible) liquid moving through a capillary, when one phase preferentially wets the capillary
surface, evidently relies on the linear velocity, \( u \), of the flowing stream, or its total flow rate \( Q \).

The linear velocities in flow injection analysis are, in general, low, and the influential flow pattern is laminar. As a result, the flow velocity near the tubing wall is zero, while in the centre of the tube it is twice the average value taken along a radius. The film forms part of a relatively stationary phase along the wall, forcing a secondary internal flow to circulate within each segment.

There is no exact theory for film formation in liquid-liquid extraction-flow injection analysis systems. However, a number of equations describing film formation in gas/liquid systems\(^{27,28}\) have been derived and also applied to liquid/liquid systems\(^{19,24,29}\). These equations have the form, previously shown in equation 1.1.

The film formed by one phase on the tubing walls as a result of a linear velocity distribution across the tubing diameter or as a result of the wetting properties of the solvent, causes changes in the geometry of the segments\(^{24,26}\). Assuming a constant volume \( V \), of the segments of one immiscible phase (in this case aqueous) in the continually flowing stream of the other phase, the lengthening of the aqueous phase segment caused by the wall film, or by changes of the nearly cylindrical shape of the segment at zero flow rate to some ellipsoidal or deformed cylindrical shape (Fig 1.3) can be predicted, the end of the segments displaying a more or less characteristic convex shape at higher flow rate\(^{24-26}\).

The segment length \( L \), can be expressed as a function of the segment volume \( V \), and the outer radius of the cylindrical segment \( r_o \), which relies on the inner radius of the tubing, \( r_i \), and the average value of the film thickness \( d_f(=r_o - d_l) \), in the form,
In this case, the segment length $L_s$ is inversely proportional to the second power of the outer radius of the segment $r_o$. With increasing film thickness, $L_s$ will increase due to the decreasing outer radius of the segment at higher flow rate or linear velocity. The relative segment lengthening decreases with increasing tube inner radius.

Assuming that the $(\eta/\gamma_w)^n$ term is constant during the studies, and by inserting the film thickness value $d_t$ from equation 1.1 into equation 1.2, the segment length can be expressed as a function of the linear velocity $u$ as:

$$L_s = \frac{V_s}{\pi (r_o - d_t u^a)^2} = \frac{L_o}{(1 - k_u u^a)^2}$$

or as a function of the total flow rate $Q_w$ in the form

$$L_s = \frac{V_s}{\pi (r_o - k_u r_q Q_w^a)^2} = \frac{L_o}{(1 - k_q Q_w^a)^2}$$

Using different values for $a$, $k_u$, $k_q$ or $k_q^1$ constants ($a = 0.1$ to $2$, $k = k^1 = 0.1$ to $1$), the theoretical effect of the linear velocity $u$ (or the total flow rate $Q_w$) on the segment length $L_s$ (or on its relative lengthening $(L_s - L_o)/L_o$) can be calculated. The resulting curves are non-linear, their shape depends upon both constants. The intercept with the $L_s$ or $(L_s - L_o)/L_o$ axis corresponds to the original segment length $L_o$ at zero linear velocity (or total flow rate $(u \rightarrow 0)$, $Q_w \rightarrow 0$), or it is equal to zero, respectively. The segment length $L_o$ can also be calculated from the originally injected volume $V_s$ of the appropriate immiscible solvent as $L_o = V_s/\pi r_o^2$. 

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The length of the aqueous segments is increased in all cases as a result of the formation of a regular film of the organic phase on tubing walls made of lipophilic material, and of the convex form of both ends of the segments.

Shorter segments are more strongly influenced by film formation, and the film is completely destroyed when very long aqueous segments alternate with relatively very short organic segments. The analytical signal measured for the aqueous segments decreases with decreasing alternating frequency of the aqueous segments, or when a single segment of organic phase is introduced into a continuous stream of water. This agrees with what is observed visually\textsuperscript{24-26} and with some experimental studies\textsuperscript{30}.

As already stated, the thickness of the film is reliant on the flow rate and flow rate ratio, as well as on the changing frequency and segment length ratio $L_{\text{long}}/L_{\text{short}}$. All experimental evidence points to power relationships with the $L_\text{r}$ values reaching a near plateau at higher flow rates $Q_\text{r}$ or linear velocities is due to a polynomial dependence of the film thickness, $d_\text{f}$, on the two factors\textsuperscript{26}. The coil material also determines film formation properties for different solvents. Materials for tubing such as polyethylene, PTFE etc., let the organic phase form a film that improves the conditions for efficient extraction\textsuperscript{24}.

The thickness of the film is generally expected to be linearly dependent on flow rate or the linear velocity, with the value of the constant ‘a’ equal to 1. These conclusions are as a result of erroneous interpretations of the experimentally found reliance of the film thickness (in microns) on the linear velocity (in centimetres per second)$^{24}$. 

Film formation also has an effect on the baseline value, as seen for segment shapes at different flow rates of the aqueous phase\textsuperscript{24}. The length
of the aqueous segment increases with increasing total flow rate \( Q_a \), and the film is destroyed at a certain defined value of the ratio of the lengths of the aqueous and organic phase segments. Above this value, the baseline is identical to that of water flow. At very high flow rates, and when the aqueous and organic segments change at a very high rate, the role of the film formation is very important. The film thickness becomes high and the baseline increases rapidly with increasing flow rate. At a very high organic flow rate with short water segments, the baseline signal cannot be compensated and segment lengths are impossible to measure\textsuperscript{19,20,26,31-33}.

At a very high total flow rate \( Q_a \), it is not possible to visually observe deviations of the flow pattern in the extraction coil, but there is a definite chance that the small organic phase segments are disrupted so that an irregular segmentation results. This may lead to poorer contact between the two phases and a decrease in the extraction efficiency. The typical physical disruption of organic phase segments has not been proven experimentally, yet it may be the cause of the observed deviations.

The ability of a segment to prevent disruption, in theory should depend on its segment length. The force that tends to disrupt the segment is proportional to the linear flow velocity. Both the segment length and linear flow velocity are inversely proportional to the cross sectional area of the tube. This means that the conditions for segment disruption will depend primarily on the tube diameter. This may explain simultaneously appearing maximum values for coils of differing internal diameter, hence, the concentration ratio cannot be increased indefinitely just by increasing sample flow rate and flow rate ratio, furthermore, at a very high total flow rate, the efficiency hence reproducibility begins to decrease\textsuperscript{34,35}. 
1.3.3 Extraction.

Liquid-liquid extraction in flow injection analysis is an effective procedure, despite having relatively large organic, and especially aqueous, phase segments. This results from the formation of a very thin near stationary film of one solvent on the inner wall of the capillary tube system, caused by the solvent having the greater affinity for the capillary coil material.

The extractable determinants are transferred from the relatively homogenous solution of the higher determinant concentration in one immiscible solvent into segments of the other phase via the segment interface by diffusion and by the internal flow within the segment. The interfacial area available for the extraction consists of menisci between the segments and the film of organic phase surrounding the aqueous phase segments in Teflon tubing, or the film of the aqueous phase surrounding the organic phase segments in glass or metallic tubing. Since the area of contact becomes very large compared to the segment volumes, the solute transfer is very efficient. The extraction equilibrium is achieved after a time delay, the time delay depending primarily upon the role of extraction and efficiency of extraction.

Two basic mass-transport processes must be distinguished—transport through the vertical interface (menisci) between the two phases (axial extraction), and transport through the horizontal interface (the wetting film) on the tubing wall (radial extraction). Which of these processes will be favoured is determined by the ratio between the interfacial area and volume, as well as by other factors. The magnitude of the interfacial contact area is influenced mainly by the segment length in the extraction coil; there is a need for the segmentation pattern to be controllable and constant during the entire procedure.
The first of these two mechanisms has an important function in normal liquid-liquid extraction-flow injection analysis using relatively short segments of both phases. When efficient segmentation is impossible or when long segments of one immiscible phase are introduced into the continuous flow of the other phase, the second mechanism plays an important role in effecting the extraction. However, as the extraction process has been found to be poor using a long aqueous sample zone with no regular segmentation, the first mechanism would predominate in liquid-liquid extraction-flow injection analysis. An integrated model of the liquid-liquid extraction may overcome the difficulties which exist connected with the two different mechanisms.

The model of liquid-liquid extraction that should be considered involves aqueous segments travelling through a surround of organic solvents on the inner surface of PTFE tubing. Extraction occurs instantaneously and determinand concentration alters slowly as a result of dispersion. It is assumed that, in a straight tube solvent extraction set-up, laminar flow occurs. The cross-sectional profile is parabolic with the linear velocity being zero at the walls, while the flow in the centre of the tubing is twice the mean value of the linear velocity. The difference between the local flow velocity drives the liquid in the segments into a circulating toroidal flow. Small eddies are produced within the segments thereby improving mixing. The model involves a species X being extracted from an aqueous segment to the organic phase, with the organic phase forming the film on the walls (Fig. 1.4). It is assumed that there is an uniform distribution of the determinand within the aqueous segment at the start of the extraction process.

Eventually, diffusion zones, i.e., the regions in both aqueous and organic segments next to the liquid-liquid junction where the movements of species X takes place, are formed. The thickness of the diffusion zones is influenced by the degree of convective mixing in the segments. In the
aqueous segment, species X is more concentrated in the bulk region (depicted in Fig 1.4 by the broken line) than in the diffusion zones.

The converse is true for the organic segments, where the concentration of X is greater in the diffusion zone than in the bulk region. In order to describe the actual extraction process, the mass transport of species X from the bulk to the interface in the aqueous segment and its transport from the interface to the bulk of the organic segments needs to be considered.

![Diagram](image)

Fig. 1.4: Schematic diagram of transport phenomena in liquid-liquid extraction-flow injection analysis manifold, $K_1$ to $K_4$ are diffusive mass transfer constants, $\beta$ are mass transfer coefficients.

Considering the kinetic process, the extraction procedure is assumed to proceed by first order kinetics. The diffuse mass transfer constants of X to and from the diffusion zone for the aqueous and organic phases (Fig 1.4) are represented by $K_1$ and $K_4$ respectively. Both are defined as $D/dZ$, where $D$ is the relevant diffusion coefficient and $dZ$ is the thickness of the diffusion layer. As a result, the greater the concentration, the smaller the $dZ$ value and the greater the $K_1$ and $K_4$
values. The rate constant for the transfer of X from the aqueous diffusion zone across the liquid junction to the organic diffusion zone is \( K_2 \). The rate constant for backward transfer is \( K_3 \).

The concentration of the determinant in the bulk regions of the aqueous and the organic phase are represented by \([X]_{\text{aq}}\) and \([X]_{\text{org}}\) respectively, while the determinant concentration in the interface is symbolized by \([X]_{\text{aqi}}\) and \([X]_{\text{orgi}}\). The interface area/volume ratio is \( S/V \). The flow across the individual interfaces can be described by \( Q_1 = K_1([X]_{\text{aq}} - [X]_{\text{aqi}}) \), \( Q_2 = K_2[X]_{\text{aqi}} - K_3[X]_{\text{orgi}} \) and \( Q_3 = K_4([X]_{\text{orgi}} - [X]_{\text{org}}) \).

At steady state conditions, \( Q_3 = Q_2 = Q_1 = (V/S) \, d[X]_{\text{org}}/dt \). Working through the equations will eliminate \([X]_{\text{aqi}}\), \([X]_{\text{aq}}\) and \([X]_{\text{orgi}}\). In the case for which \( V_{\text{aq}} = V_{\text{org}} \), i.e., where the volume of both phases is equal, this gives:

\[
1 - \left( 1 + \frac{1}{K_d} \right) \left[ \frac{[X]_{\text{org}}}{C} \right] = \exp \left( -t \left( \frac{S}{V} \right) \right) \frac{K_4(K_2 + K_3)}{(K_1K_3 + K_1K_4 + K_2K_d)}
\]

The distribution constant \( K_d \) is equal to \( K_2/K_3 \). Residence time in the extraction passageway from phase segmentation to phase separation is represented by \( t_o \), while \( C \) is the initial concentration in the aqueous phase. When mass transport to the interface (\( K_1 \) and \( K_d \)) is fast enough, compared to the transport across the interface, several terms can be eliminated, and

\[
1 - \left( 1 + \frac{1}{K_d} \right) \left[ \frac{[X]_{\text{org}}}{C} \right] = \exp \left( -t \left( \frac{S}{V} \right) \left( K_2 + K_3 \right) \right)
\]

Although this simple model does not allow for all the possible kinetic aspects of an extraction, it can be concluded that the ratio between the interface area and the phase volume is very important.
When applied in practice the following points are important:

- The rate of extraction is vastly improved by increasing the (S/V) ratio; by decreasing the inner tubing diameter a maximum surface contact between phases can be achieved, thereby increasing the probability of the analyte to cross.

- Mass transfer to and from the interface increases with flow rate, possibly due to an increase in the convective constituent of intra-segmental mixing.

- The rate of extraction is also increased by decreasing the segment length, not so much due to an increase in boundary areas but rather largely attributed to eddies formed at points on the tubing walls; the number of eddy points increases with the number of segments per unit extraction passageway length.

In coiled tubes, however, the role of a secondary flow is taken into consideration. Centrifugal force is produced as a result of the helical geometry of a coil at a maximum on the fastest moving regions of the flow at the tube centre. This portion of the fluid is transported radially outward and replaced by fluid flowing tangentially along the wall. This secondary flow allows intra-segmental mixing in coiled tubes to occur more efficiently, so that mass transfer is increased. This process can be correlated with the dimensionless velocity function $De^2Sc$ where:

$$De^2Sc = \left[ \frac{(d_i^3u^2)}{(nD)} \right] \left[ \frac{1}{d_c} \right]$$

1.7

in which De and Sc are dimensionless Dean and Schmidt numbers, $d_i$ is the inner diameter of the tubing, $u$ is the average linear velocity of the fluid, $n$ is the kinematic viscosity, $D$ is the diffusion coefficient of the solute, and $d_c$ is the coil diameter measured at the tube axis.
Similar to the previous model for straight tubes, extraction in coiled tubes is treated as a first-order kinetic process and assumed to be controlled by mass transfer to and from the aqueous/organic interface\textsuperscript{26}. The integrated form of the rate of extraction expression is
\[
\ln\left(\frac{A_{eq,o}}{A_{eq,o} - A_{t,o}}\right) = K_{obs}t
\]
where \(A_{eq,o}\) and \(A_{t,o}\) are either the steady-state analyte absorbances or peak areas, due to the determinant in the organic phase at equilibrium and at time \(t\). The observed extraction rate constant \(K_{obs}\) is a function of both the mass transfer with the individual segments and the interface area \((S)\). It is related to \(S/V\), where \(V\) represents the volume of the segments, and its ratio as \(K_{obs} = (S/V)\beta\), where \(\beta\) is the overall log mean mass transfer coefficient and has the unit of velocity, and its reciprocal is often regarded as the resistance to mass transfer.

This coefficient is related to individual mass transfer coefficients by the expression
\[
\beta = \left\{\frac{1}{\beta_{aq}} + \frac{1}{\beta_{org} K_d} + \frac{1}{\beta_i}\right\}^{-1},
\]
where \(\beta_{aq}\) and \(\beta_{org}\) refer to mass transfer coefficients to the interface through the aqueous phase, and away from the interface through the aqueous phase, and away from the interface through the organic phase, respectively. \(K_d\) is the distribution coefficient of the solute, and \(\beta_i\) refers to the transfer coefficient across the interface itself (Fig. 1.4). In the absence of surface-active solutes, the \(1/\beta_i\) term can be neglected. In liquid-liquid extraction-flow injection analysis manifolds, \(K_d\) is usually large, meaning that \(\beta_{org} K_d\) is much greater than \(\beta_{aq}\) and the organic phase acts as a "sink" for the solute. Therefore, the extraction rate is controlled by mass transfer within the aqueous phase only where \(\beta = \beta_{aq}\).

As the solute leaves the aqueous phase the sides and both ends of the aqueous segment, \(\beta_{aq}\) can be treated as the sum of the products of aqueous phase mass transfer coefficients, one for axial mass transfer to the ends, one for radial mass transfer to the sides of the aqueous segments, as
From experimental data, some general trends are observed:

1. The rate of extraction is enhanced by a high \( S/V \) ratio, best achieved with a small tubing diameter.

2. The extraction rate increases with decreasing segment length. Similar to the model for straight tube extraction, the increase in \( S \) accompanying a decrease in segment length accounts for only a small fraction of the increase in \( K_{\text{obs}} \). This phenomena can be explained in terms of \( \beta_{\text{aq}} \). When the segment is sufficiently reduced that the ends of the segment are of the order of a tube diameter apart, they interact hydrodynamically to increase the radial velocity component of the circulation within the segment. In effect, \( \beta_{\text{aq}} \) increases as convective intra-segmental mixing increases.

3. Increasing the flow rate increases the rate of extraction with respect to time, brought about by the increase in intra-segmental mixing. Although a corresponding increase in the thickness of the stationary film is also expected, the mass transfer rate is not effected since the rate determining mass transfer process takes place in the non-film forming aqueous phase.

4. Based on equation 1.8, the tighter the coil, the faster the secondary flow and therefore the greater the mass transfer. This effect is of greater significance for long segments than for short segments due to the smaller velocity differential of short segments in a coiled tube. Unlike long segments, the axial flow profile in short segments is less than parabolic, hence, the centrifugal forces are uniformly distributed across the tube.
From the two models discussed it can be seen that they have a number of common aspects. They cover two extreme situations existing in liquid-liquid extraction-flow injection analysis systems, associated with the use of tubes of different geometry. It is obvious that an increasing need exists to formulate more general models covering the intermediate situations of flow injection analysis. Such models will probably describe all of the possible segment geometries, fast to slow chemical reactions that occur, and differences in the extraction rates. It should be considered that the main part of the extraction process takes place in the extraction coil. However, some part of the extraction takes place in the segmenter and phase separator, where relatively intense mixing of both phases takes place as a result of numerous factors, such as differences in manifold geometry, changes in character of the flow through the phase separator and segmenter, changes in the space orientation of the flows and flow rates.

The enrichment factor, i.e., the ratio between the flow rates of the aqueous phase containing the sample and organic phase, is effected by the segmentation process. The enrichment factor rarely exceeds a value of twenty in most commonly used manifolds. The length of the organic and aqueous phase segments cannot be reduced indefinitely. The lower limit of the organic phase segment length has been experimentally estimated to be one and one half times the integral diameter of the extraction coil tube\(^24\). The extraction efficiency decreases dramatically if the droplets of the organic phase become too small to form and maintain a continuous film on the tubing wall. Segmentation may be unstable due to mixing of very small segments during transport through the extraction coil.

The efficiency of the reaction or extraction coil can be expressed in the following form:\(^{40}\)
\[ m = \frac{Q_o}{1000} \int C_o \, dt \]

\[ = Q_o \text{ constant} \int A \, dt \]

where \( m \) is the number of moles extracted, \( C_o \) is the molar concentration of the sample component extracted into the organic phase passing through the membrane and flow cell and \( A \) is the absorbance (when \( C_o \) is calculated from the measured absorbance \( A \), path length \( l \), and molar absorptivity \( \epsilon \), where \( C_o = A/l\epsilon \)). The peak area is defined as

\[ A_p = A_1 \cdot H_p \cdot W_p \]

where \( A_1 \) is the absorbance corresponding to 1 cm of the peak height \( H_p \) and \( W_p \) is the peak width. The apparent extraction efficiency \( E(\%) \) can be expressed as \( E(\%) = (100m/C_o)\times V_s \), where \( E(\%) \) is the percent of determinant extracted, \( C_o \) is the concentration of the analyte in the sample solution (mol/l), and \( V_s \) is the volume of sample injected. The efficiency of extraction and peak height obtained using narrow tubing are better than those obtained with larger bore tubing\(^{40}\).

In some cases, the segmentation can breakdown at higher flow ratios\(^{41}\). Segmentation breakdown is more likely for phases which form thick films than for those forming thin films. The thicker film depletes the segments of organic phase, making them shorter and more likely to be disrupted.

1.3.4 Dispersion.

An injected aqueous analyte is not only present in the injected segment and in an adjacent segment of organic phase, but, to some extent, also downstream (and partly upstream) from the injected aqueous segment. Hence, liquid-liquid extraction does not only occur at the boundary between the organic/aqueous phases containing the sample. The
segments of an immiscible fluid and of an aqueous sample injected into a segmented flow do not remove the dispersion since inter-segmental transfer and mixing occur.

It is known for liquid-liquid extraction-flow injection analysis manifolds, that using different organic phases and tubing materials can greatly affect the dispersion. In some cases e.g., chloroform and water in Teflon tubing, using low to medium flow rates, no dispersion of the sample is observed, even when long extraction coils have been studied. Other causes of analyte dispersion such as adsorption on the tubing walls should be noted.

Each part of the liquid-liquid extraction-flow injection analysis manifold participates in the overall dispersion. The idea of dispersion has to be applied to all components and functions taking place which alter the concentration profile of the sample. The overall dispersion consists of partial terms connected with the components.

When, the analyte is present only in the organic phase, and the aim is to transport it with minimum dispersion, segmentation of the organic phase with water is suggested when using metal or glass tubes. This means that the aqueous layer will form a film, encapsulating the organic phase segments so that no inter-segment mass transfer takes place. In liquid-liquid extraction-flow injection analysis, it has been reported that the transport through the extraction coil of a solute dissolved in the film forming phase segments, is produced such that there is band broadening of the peak. However, if the solute is in the non-film forming liquid, minimal peak dispersion is seen.

The dispersion procedure is less significant for thinner films, thus the dispersion decreases with decreasing flow rate as narrower peaks are observed. The coil material influences the dispersion process since
PTFE or polyethylene tubing allows the organic phase to form a thick film which improves extraction efficiency.

Another important way to reduce the dispersion is to increase the exchange between the stationary layer and the bulk of the segments. To further this, the inner diameter of the extraction coil should be small, thereby giving a short diffusion distance between the stationary layer and the bulk of segment.

Therefore, physical evidence of a film, studies of the conditions under which it occurs, and useful approaches for measuring the film thickness are of crucial importance to increasing the understanding of the dispersion phenomena in segmented liquid-liquid extraction manifolds.

In the extraction coil, two different types of mass transfer from one segment to the next segment of the same phase may be distinguished. A solute in the phase forming a thin film on the tube walls can then be dispersed while it is moving through the extraction coil by inter-segmental mixing. The quantity of solute which is moved by the film determines the size of dispersion, and therefore the thickness of the film is a major factor.

The extraction procedure can be rapid so the species originally present in a segment at a high concentration is transported across the interposed segment of the other phase into the next segment of the same phase, causing band broadening. The film has an important function in the analyte peak broadening procedure, i.e., in the dispersion and dilution process of the sample. The thicker the film, the greater the peak broadening.

When an extraction is carried out in a flow injection analysis manifold, designed so that the extraction coil is just long enough to attain
equilibrium, the extractant will be present in both phases approximately half its time. This means that for a fixed organic phase the tubing material should be chosen in such a way that the phase forming the thinnest film "wets" the tubing surface. When the organic phase is stabilised, so is the surface tension, implying that the tubing material should be chosen such that the phase with the lowest viscosity wets the tubing walls.

The most crucial component with respect to the total dispersion is the phase separator. There is a near linear relationship between analyte peak height and fraction of organic phase passed through the flow-through detector. This emphasizes the importance of an efficient phase separator.

Therefore, when the proportion of organic phase passing through the flow cell is decreased, the analyte peak height also decreases. As a result, the proportion of the sample reaching the detector cell is decreased (similar to one-phase flow injection analysis). If analyte concentration is being measured, a decrease in the proportion of the organic phase does not necessarily lead to a decrease in sensitivity if the dimensions and dispersion of the detection system is decreased in turn.

The volume of the phase separator probably cannot be made smaller than twice that of the sample volume. In a system when liquid-liquid extraction is allowed to reach equilibrium, fluctuations in the segment length are unlikely to change the analytical precision. On the other hand, in a system where the extraction coil is short so that extraction is incomplete, the precision of the procedure can be influenced by fluctuations in segment length.

For shorter tubes, the use of tubing of 0.5mm internal diameter gives a stronger signal than that seen with 0.8mm internal diameter tubing. An
explanation of this is that there is a greater efficiency of the liquid-liquid extraction process with lower internal diameter tubing, which is as a result of both convective flow patterns and the organic phase film on the walls of the tubing.\textsuperscript{24,38,39,44}

1.4 Components.

Several basic factors have to be considered when designing a liquid-liquid extraction-flow injection analysis manifold. These are concerned with reproducible segmentation of the immiscible phases; optimization of the geometry of the complete liquid-liquid extraction manifold; the choice of suitable materials for the construction of the individual components; and a highly efficient and fast separation of the phases after reaching separation equilibrium.

All of the individual precautions are important for optimum sensitivity and lowest possible zone broadening in sample analysis. Separation and segmentation of the two immiscible phases are the most important steps, but are also the most difficult processes of this technique.

Most components of liquid-liquid extraction-flow injection analysis are commonly used in a classical one-phase flow injection analysis manifold. The exceptions are the segmenter, the extraction coil and the phase separator. Comprehensive reviews are reported elsewhere.\textsuperscript{9-12} Therefore, only the components exclusive to liquid-liquid extraction manifolds are described. Special modifications of detection systems and appropriate interfaces are also mentioned.

All components used in liquid-liquid extraction-flow injection analysis should be selected with emphasis placed on the aggressive properties of the organic solvents used in the extraction process, as many solvents damage materials used in ordinary flow injection analysis. Therefore, all components and tubing should be of chemically inert material e.g., fluoroplastic, glass, platinum,
titanium or stainless steel. Components which would normally be used in both one-phase and liquid-liquid extraction-flow injection analysis need not be specially constructed for liquid-liquid extraction-flow injection analysis as they serve the same purpose in both processes.

The choice of suitable detection systems is restricted by the presence of trace concentrations of one of the solvent phases in the other, as the solubility of solvents is usually quite low but measurable. Optical detection is preferred to electrochemical detection, with the effect of the organic phase being less important for the former.

1.4.1 Transport units.

An aqueous stream can be produced by using a standard peristaltic pump with ordinary PVC tubing, or several other techniques\(^{8-11}\). In contrast, a flow of organic solvent is normally produced by:

1. A liquid chromatographic pump, piston or syringe, with pulse dampers and pressure indicators.

2. A displacement technique, involving pumping an aqueous stream into a closed container (usually thick-walled bottle) by using a peristaltic pump with ordinary pumping tubes, the container is completely filled with an organic solvent, which is replaced at a constant flow rate of aqueous phase and not forced into the flow injection analysis manifold (Fig. 1.5).

3. A peristaltic pump with the manifold pump tubing constructed of inert material e.g., modified PVC, silicone rubber, flexible polyurethane; fluoroplastics etc., since the flexible tubes normally used in flow injection analysis are rapidly destroyed.

26
Fig. 1.5: Two possible applications of the liquid/liquid displacement technique for an organic solvent being heavier (A) or lighter (B) than water and for the constant air pressure displacement technique (A). Deaeration channel is optional.

4. A constant gas overpressure (using pressurised inert gas) forcing the organic phase through the flow injection analysis system from a closed container.

A stable pumping system is normally needed in liquid-liquid extraction-flow injection analysis. Rigorous control of the flow rate is sometimes not possible with peristaltic pumps as non polar solvents destroy normal, inexpensive commercial solvent-resistant tubing e.g., Viton, Acidflex tend to degrade with time. Small particles from the flaking of the inside of the tubing walls may clog the porous membrane of the phase separator.

Basically, the flow should stop and start instantaneously for precise control of the flow of the stream, which is important more so for intermittent pumping parameters. A small inner "hold up" volume is required to allow rapid start up and short wash out times. The greatest
disadvantage of reciprocal pumps is pulsing of the stream. The flow rate alters significantly during the starting period of the peristaltic pump, in order to obtain a constant flow some form of conditioning of the pump tubing is needed.\textsuperscript{9-12}

High performance liquid chromatography pumps are a rather expensive solution to the problem, especially when several independent solvent flows are required; however they cannot be used with a low pressure manifold without introducing an additional column to yield a necessary overpressure for the proper function. Syringe pumps are extremely expensive. However, a well defined segmentation pattern may be created through the use of alternately operated stepper-motor-driven microsyringe pumps.\textsuperscript{15} Chemically inert circulating pumps with a small dead volume are not as yet commercially available.

Displacement techniques, using either aqueous phase pumping or constant gas pressure, can in many cases offer less expensive and better alternatives. Membranes, made of chemically inert materials or a thin layer of an insoluble substance, can be used to prevent or minimize the solubility of the gas or water in the organic solvent. It is sometimes difficult to obtain a constant flow rate through an extraction flow injection analysis manifold using constant gas pressure since small variations in the flow rate may result in tube clogging or pressure variations in one of the containers.

1.4.2 Segmenters.

Phase segmentation involves dividing the continuous flows of the organic and the aqueous phase into one uniform stream with alternating segments. The immiscible phases are brought together in a narrow tube in a controlled manner so that defined segments of each phase are formed. Segmentation and separation of two immiscible phases is of vital importance to the quality of the results obtained. Detailed studies of
parameters controlling segment size and reproducibility of segmentation have focused on segmentation of the gravity/density and hydrodynamic types$^{26,31,39,46,47}$.

Two important variables need to be considered when dealing with segmentation; segmentation reproducibility and size of segment. Although the size of the segment may not affect the efficiency of extraction of a rapid extraction process or when large sample volumes are introduced into the system, it could theoretically change the efficiency of a slower system$^{21,39}$. The maximum size of the segment is determined by the interfacial tension of the organic and aqueous phases, both between each other and between a phase and the tubing material, such that the maximum segment size decreases with decreasing values of interfacial tension. The choice of tubing material, dimensions of tubing and the geometry of the mixing chamber are also very important. The ability to vary the length of the segment is limited with most of the segmenters available.

Accurate performance of the segmenter is important for the successful operation of the liquid-liquid extraction manifold. The overall segmentation process can negatively effect sample dispersion, rate of extraction, and phase segmentation$^{21,39}$. Reproducibility of segmentation is often not ideal, and the function of segmenters at high flow rates and phase flow rate ratios necessary for sample work-up is often limited. Reproducible segmentation can improve the precision of signal measurements, and may simplify data processing. It may even be possible to dispense of phase separation, especially if the segmentation reproducibility is enough to allow precise timing of the measurement intervals.

Several segmenter types of varying efficiency have been reported in the literature. The most common segmenter types are T-piece (Fig. 1.6) segmenters made of glass$^{48,49}$, fluoroplastic$^{13,22,30}$, stainless steel$^{51}$ or glass
lined T-pieces of stainless steel, and combinations of hydrophobic and hydrophilic materials\textsuperscript{52,53}.

Fig. 1.6: Classical T-pieces (A,B,C) with the most often used orientation of the aqueous, organic and segmented flows, but other orientations are also possible.

Improved glass A8-T (Fig. 1.7) and A10-T\textsuperscript{49,54-56} and T-pieces (Fig. 1.8) constructed from fluoroplastics with inserts of fluoropolymer tubing\textsuperscript{50,57-60} or with an enlarged inner diameter of the outflow channel\textsuperscript{39,60,61} have found a wide range of applications.

Fig. 1.7: Improved A8-T fitting with glass and platinum inlet capillaries and two fluoropolymer tubing inserts in an outflow channel (with two possible positions A,B) and the A8-T fitting in the reverse position (C).
Fig. 1.8: An improved glass T-piece with fluoropolymer tubing inserts (A), a fluoropolymer T-shape segmenter with an enlarged outflow channel (B).

Differing configurations of Y- or W pieces (Fig. 1.9) made of glass or fluoroplastics and four-way fittings have also been recommended.

Fig. 1.9: Y-piece (A,B,C), W-piece (D,E) and four-way (F) segmenters with the most often used orientation of the aqueous, organic and segmented flows, but other orientations are also possible.
Recently, a coaxial, falling drop (Fig. 1.10) segmenter has been introduced\textsuperscript{20,26,32-34,69} in an attempt to overcome some of the disadvantages of other segmenter types.

The geometry of the inner capillary system of the T-shape segmenter\textsuperscript{40,47,48} and the geometry of the mixing compartment of the coaxial segmenters has also been investigated\textsuperscript{31}. Flow systems without segmentation\textsuperscript{41,42,61,70-73} and the use of sample introduction downstream from the segmenter have been suggested\textsuperscript{38} since extraction efficiency, rate of extraction, and sample dispersion (peak broadening) are all influenced by the segmentation pattern. In addition, a multichannel dropping segmenter has been used for homogenization and the instantaneous introduction of samples and reagent solutions into the continuous flow of the other immiscible solvent\textsuperscript{19,20,26} directly inside the segmenter.

Other studies have shown that conventional loop injectors operated by a cycling motor with adjustable filling and draining times and a brief intermittent period can be used at low flow rates or for introducing very long segments\textsuperscript{19,74} of an immiscible solvent into the continuous flow of the other phase.

Segmenters can therefore be classed, depending on the basis of segmentation (post segmenter introduction of the sample being a special case) as either continuous flow segmenters or mechanical type segmenters.
Fig. 1.10: Coaxial segmenters of different geometries made of polyvinylidifluoride (PVDF) or Perspex (A), PVDF body with a thick wall glass mixing chamber and PVDF conical chamber with reverse-orientated phase flows a PVDF screw with a single- (B) or a multiple- channel inlet system (C) for continuous introduction of aqueous stream(s), an all glass segmenter (D), and a glass segmenter with two different geometries of the PVDF conical compartment (E,F).
1.4.3 Extraction coils.

There are two basic considerations to be made when choosing an extraction coil: the material the coil is made from and the coil dimensions. When considering the material of the coil, the question arises of whether to use hydrophilic (glass or metal capillary) or lipophilic (fluoroplastics) materials. The choice depends upon whether the determinant is to be extracted from the aqueous into the organic phase or vice versa. The former case is the procedure used mainly in liquid-liquid extraction manifolds, whereas the latter is normally used in two stage extraction processes. The material of the coil can indirectly affect the efficiency of extraction and the sample dispersion, by changing the thickness of the film.

The second consideration, with respect to the coil dimensions, can influence sample dispersion and efficiency of extraction, as related to kinetic efficiency, total extraction yield, and peak broadening. The liquid-liquid extraction process requires that the segmented phases remain in contact, while a state of thermodynamic equilibrium is reached by the analyte in partitioning between the two phases. The extraction coil can be made long enough in order that transfer kinetics do not represent a limiting factor in the efficiency of extraction. Any increase in coil length above this point causes an increase in sample dispersion without any accompanying rise in sample recovery and is therefore disadvantageous.

The extraction coil is normally a relatively narrow bore teflon tube i.e., 0.2 to 1.5 mm, several centimetres to several metres in length. The geometry of the coil, the quality of the inner wall surface and the spatial orientation affect the rate of extraction, the efficiency of extraction, and the yield of the extraction process. The coil should be free of any sharp changes of inner diameter and sharp bends and edges. Its inner surface should be smooth and completely free of deposits that may alter the hydrophobic character of the tube material.
Extraction coils have been used in various configurations of flexible Teflon tubing, but the simplest and the most frequently used coils have a symmetric or helical shape with a coil diameter ranging from units to tens of centimetres. The horizontal position of the main axis of the coil forces mixing, and introduces a secondary flow rate perpendicularly arranged to the axial, this increases mass transport.

The secondary flow patterns are established in response to centrifugal force in coiled capillaries. They bisect the capillary profile, decreasing the diffusion distance by one half. The radial secondary flow causes effective mass transport, interchanging material in a slower moving streamline with material in a faster one\textsuperscript{10}. Therefore, mass transport is forced by intra-segmental movement as a result of the viscous drag of the two immiscible fluids. Curved and coiled extraction coils should, therefore, give improved radial mixing in the segments and result in a more effective extraction.

The secondary flow in a coiled tube is disrupted when its coiling radius or its position are altered, and as a result the mechanical forces acting on sample dispersion are changed. It should be considered that a coil diameter of up to 60 or 80 mm does not drastically influence the analytical signal\textsuperscript{15,75}. The intense secondary flow can sometimes negatively influence the efficiency of extraction.

Coils containing glass or teflon beads or glass wool plugs, or which are subjected to high frequent agitation have been shown to enhance contact between the two phases in flow analysis manifolds. Additional methods e.g., immersion of the coil in a thermostatic bath maintained at elevated temperatures, or subjecting the coil to an ultrasonic field, have also been used to increase mass transfer efficiency. The use of knitted (or even knotted) open tubular coils, which effectively reduce axial dispersion in homogeneous systems, have been found to decrease band broadening for
some single phase flow injection analysis applications\textsuperscript{9,10}. Convoluted coil configurations with small turn radii e.g., serpentine reactor, severely disrupt laminar flow and can be potentially more efficient than a simple coil\textsuperscript{9,10}. However, liquid-liquid extraction in a coiled tube is as effective in reducing band broadening as a knitted open tube of the same internal diameter as the average peak variances show no statistical difference\textsuperscript{9,10}.

At short or zero residence times, the relative peak area is always less in a straight tube than in a coiled or knitted tube as some limited time is necessary for equilibrium to be established between the two phases. There is no obvious difference between coiled and knitted tubes, indicating no significant difference in the efficiency of extraction. In addition, there is no band broadening reported. The coiled tube is easier to use and has a lower back pressure than other configurations. A straight tube is the easier and most often used configuration when a very short residence time is necessary. In some cases segmentation reproducibility and analytical signal functions are preferred for straight tubing than for coils\textsuperscript{20}.

The difference in viscosity of the two immiscible solvents shows itself in the extraction process as an inhibitor of diffusion. With increasing viscosity, the rate of mass transfer decreases, thereby reducing radial mixing and increasing the residence time of the sample component(s).

Length of the segment in the extraction coil is the main factor controlling the size of the area of contact between the two phases. A disordered segmentation pattern causes losses of solvent by the wetting process due to the interfacial film thickness constantly changing. Serious mixing of segments of varying sizes is due to differences in the linear velocity of segments having different geometries due to differences in the viscous drag of the wetting phase. This implies that the segmentation pattern needs to be controlled and kept constant during the entire analytical procedure.
1.4.4 The phase separator.

This is the last essential element of a liquid-liquid extraction manifold and probably the most relevant to appropriate functioning. The phase separator receives the segmented liquid-liquid flow from the extraction coil and separates the segments in such a way that two independent streams of the two phases are obtained at its exit. Complete phase separation is accomplished only rarely. Therefore, the actual function of the phase separator is to provide a flow of one phase containing none of the other; this phase should be the one containing the determinand or its reaction product.

The phase separator has to operate highly efficiently and quickly so as not to minimize the overall sampling frequency. Furthermore, it should not contribute to the dispersion or dilution of the determinand, i.e., its inner volume should be as small as possible. In addition, its function should be smooth and consistent throughout the extraction procedure in order to obtain reproducible results. This requires controlling of the flow rate of the emerging phase of interest.

There is a large variety of phase separators. Nearly, every research group working in the area develops their own unit. However, they can be classified into three broad categories according to their operational principle, which also dictates their internal shape.

Separators based on density differences.

This is the simplest form of separator. A mini-chamber receives the segmented flow from the top or through one of its sides. A single interface is produced due to density differences, the flow of the heavier phase leaves the mini-chamber from the bottom, while the lighter phase leaves from the top or from one side. This type of phase separator is very similar in design to a separatory funnel, with significant differences of miniaturization and length of time needed to complete the separation.
Various models of this type of phase separator are shown in Fig. 1.11.

![Diagram of phase separators](image)

**Fig. 1.11: Different types of gravity-based separators.**

An open design is depicted in Fig. 1.11A, in addition to separating the segmented flow, it must also act as a debubbler; phase separation is achieved by an overflow system which constantly removes the lighter phase while the heavier phase is transported to the detector. A similar design made of glass has been reported by Fossey. The other two designs in Fig. 1.11 are of the closed type. In Fig. 1.11B, the lighter phase emerges from the same port through which the segmented flow was originally introduced; alternatively, in the design shown in Fig. 1.11C, the segmented flow enters on one side and the heavier phase leaves the phase separator through the other side, while the lighter phase emerges from the top.

The fact that the phase separators in Fig. 1.11B and Fig. 1.11C do not exploit wettability may account for their poorer results in terms of reproducibility. Furthermore, dispersion is rather high due to the large volume of the mini-chamber. As a result of these shortcomings these phase separators are rather outdated.
T-type separators.
In this form of separator, the segmented flow enters through one side and the phases are continuously separated at their mixing point, the heavier phase emerges from the base, while the lighter phase leaves the chamber at the top. This type of separator is made from A-4 and A-10 Technicon connectors, the efficiency of which is improved by placing small strips of Teflon or hydrophobic paper into the tube and through which the organic phase emerges, this increases the process efficiency by wettability in addition to gravity differences. The scheme of a T-type separator is shown in Fig. 1.12; in this instance, the organic phase is the lighter, so the Teflon insert points to the top of the T.

![Scheme of a T-type separator](image)

**Fig. 1.12: Scheme of a T-type separator.**

Membrane separators
This type of separator is similar to most gas-diffusion and dialysis units as it is based on the use of a semi-permeable membrane that is wetted selectively by one phase. Depending on how the outgoing flows are orientated, density differences can also be exploited. The main difference between this type of segmenter and dialyser and gas diffusers is that it separates whole phases rather than analytes or their reaction products.
Membrane phase separators normally consist of two blocks of KEL-F, Teflon, Daiflon or Perspex with holes that allow entry of the segmented flow and exit of the organic and aqueous flows. In addition, the blocks also have threaded holes for connections to be made within. Their central zone, is where the three channels meet, and is constructed in such a way that it provides a mini-chamber with a volume of 30-100\mu l. The membrane is placed between the two blocks, establishing two chambers, it does not matter if these channels are not of equal volume. To avoid leakage, the two blocks are secured with four to eight screws, occasionally two metal plates are also applied to the sides of the blocks to help the separator retain its shape.

This type of separator usually uses hydrophobic membranes which are only permeable to the organic solvent, the organic phase is usually the one of interest and should be free of aqueous phase for suitable monitoring. The membranes most frequently used in this respect are made of polyethylene backed Teflon and Fluoropore filters with a pore diameter between 0.2 and 1 \mu m.

The ability of a membrane phase separator to exclude water depends upon intrusion pressure. This in turn depends upon:

(a) the pore size of the membrane e.g., 2.8 bar at 0.2\mu m and 0.7 bar at 1.0\mu m.

(b) the hydrophobicity of the organic phase (the higher the hydrophobicity is, the higher the pressure will be); and

(c) the pressure of substances that can be absorbed by the membrane and thus decrease the pressure.

Hydrophilic membranes are much less commonly used for this purpose. They consist of one or several layers of filter paper e.g., Whatman no. 5, Whatman IPS.
To prevent difficulties arising from the physical instability of an unsupported membrane zone i.e., the active zone in phase separation, an inert Teflon support is used. This support can be a \(-1\) mm thick sheet of PTFE, a perforated Teflon strip, or a commercially available (Millipore) Teflon-coated stainless steel filter. In this last case, the filter thickness should be consistent with the design of the separator blocks. The support is necessary when considering large active membrane zones, as is the case with cylindrical separators or when high pressures are used e.g., use of a phase separator as a post column reactor in hplc.

The efficiency of membrane phase separators can be increased by slightly increasing the overpressure on the channel through which the phase that is not completely separated emerges, normally the channel for the aqueous phase and some organic, this channel should be connected to the segmented entry flow. Therefore, with hydrophobic membranes, the exit channel for the aqueous phase is provided with a restrictor coil to achieve the overpressure and allow passage of the organic phase across the membrane. Alternatively, the exit flow rate i.e., organic phase can also be controlled by additional pumping.

Only two models of membrane separators (Fig. 1.13) will be described. As explained previously most research groups design their own. Since the organic phase is normally that which should be free of the aqueous phase, a hydrophilic membrane is used and the flow that crosses it leaves through the opposite side to the segmented flow entry.
Grooved phase separators (Fig. 1.13A) are the simplest and earliest used in flow injection analysis\textsuperscript{22,65,73}. The two plastic blocks fit through several profiles and squeeze the membrane in between. Both blocks have central grooves that give rise to mini-chambers with inner volumes of between 3.5 to 500 µl. As with dialyzers, there are also some separators with widening grooves shaped like Roman frets\textsuperscript{38}, symmetrical conical cavities and flat coils (commercially available Tecator model). A large active separation zone should be obtained in order to increase the efficiency of the process; however, the inner volume should be as small as possible in order to avoid dispersion or dilution of the analyte and hence accurate signals. Usually a compromise has to be reached between these two aims, with a suitable mini-chamber design. The effect of the volume and shape of each of the mini-chambers separated by the membrane on dispersion has been reported to be different\textsuperscript{78}; the result being separators with asymmetric chambers. The most suitable results are
obtained by changing the region through which the segmented flow enters and the unmonitored aqueous plus organic phase leave. The way in which the entry and exit channels are arranged also affects the separation efficiency, however the difference between lateral, central and angled entry or exit is usually insignificant. Nevertheless, the arrangement in Fig. 1.13A is one of the most efficient in this respect as it provides smooth, long term and efficient separations.

The active zone of cylindrical cavity-type phase separators (Fig. 1.13B) is circular. This larger separation surface results in an increased efficiency. Difficulties arising from the mechanical stability of the membrane are prevented by using a suitable inert support. The areas on each side of the membrane are usually different. The mini-chamber receiving the segmented flow and providing an exit to the aqueous plus some organic phase can be parallel to each other, at an angle of 45° or 90°, or conical (Fig. 1.13B)13. This is also true of the mini-chamber providing an exit for the organic flow.

Whenever the two phases are monitored, after liquid-liquid extraction, simultaneously the separator has to provide two completely 'clean' phases. This can be achieved by using a dual-membrane phase separator (Fig. 1.14)65,79,80. The segmented flow enters the central mini-chamber laterally; this contains a hydrophilic and hydrophobic membrane which separates the cavities through which the aqueous and organic phases leave. Since extraction may not be complete, there is an exit flow to waste. Therefore, unlike other phase separators, this unit has one entry and three exits.
In 1987, De Ruiter et al. reported a membraneless phase separator (Fig. 1.15). Its operation relies on the two principles of selective wetting and differences in the phase density.

One of the blocks and the central Teflon disk have engraved grooves that bind the separation mini-chamber (8-43 μl inner volume). This has three connections: one for entry, which is located on one end and receives the segmented flow, and two for exit of the aqueous (lighter) and organic (heavier) phase. In short, this type of separator is similar to a membrane separator in appearance whereas its function resembles that of a T-type separator.

Membrane phase separators have also been used to carry out mixed dialysis/liquid-liquid extraction processes by using special membranes to separate two immiscible liquids continuously and directly reaching the separation unit. It is the analytes rather than the liquid phases that are transferred across the membrane in this case.\textsuperscript{41,65}
Compared to other types of phase separator, membrane separators show a number of advantages, namely:

(a) higher efficiency in phase separation i.e. typically 90-95%, but even 100% if some technical modifications are introduced;

(b) they can be used with a variety of organic immiscible solvents as no substantial density differences with the aqueous phase are needed;

(c) they result in smaller dispersion and dilution of the analyte as a result of the small separation chamber;

(d) they can be used over a much wider range of flow rate ratios, which results in increased pre-concentration capacity; and

(e) they can be used with high overall, i.e., organic plus aqueous phase, flow rates which gives increased sample throughput.

1.4.5 Detectors and interfacing.

A suitable flow-through detector should have the following characteristics: small volume, low noise levels, fast linear response over a wide concentration range, and high sensitivity. Other important aspects include the detection limit and the detector contribution to the peak.
The selection of adequate detection systems is limited by the presence of trace amounts of one of the immiscible solvents in the other phase since the solubility of solvents is normally quite low but not negligible. Although classical detection systems with excellent precision have been used, optical detector systems are preferred to other types as the effect of the organic phase is less crucial for such systems. Electrochemical detectors are normally not used due to the interference of trace concentrations of organic solvents in the aqueous phase.

As most liquid-liquid extraction processes, especially those using liquid-liquid extraction of metal chelates with organic analytical reagents, are non-selective, liquid-liquid extraction is often combined with relatively selective detection systems. Atomic absorption spectrometry, atomic emission spectrometry, inductively coupled plasma optical emission spectrometry, fluorimetry, chemiluminescence and especially molecular absorption spectrometry are the most popular. The simplicity with which liquid-liquid extraction-flow injection analysis can be interconnected with a flow cell means that these methods can be used to their advantage.

A detection system with two independent detectors has been used for the simultaneous monitoring of both immiscible phases after their separation using two membrane separators i.e., lipophilic and hydrophilic, connected in series, or with the aid of a dual membrane separator in which the organic phase passes through a Teflon membrane and the aqueous phase passed through a filter paper membrane. Simultaneously, the phases are directed to the detectors, and the analytical signals are fed into digital integrators.

To decrease the complexity of the manifold both phases have been monitored by a single detector (Fig. 1.16) in which the organic phase flows through the sample flow cell and the aqueous phase stream flows through the reference flow cell.
Fig. 1.16: A flow injection extraction manifold with a dual phase separator and a single detector for simultaneous monitoring of both phases passing through sample and reference flow cells.

A single photometric double-beam detector was modified to allow electronic switching of the sample/reference designation of both flow cells by use of a "double-throw relay". The setting of this relay is controlled with an electronic timer after a preselected time delay, to measure both phases as positive signals.

The assembly of two spectrophotometric detectors has also been used for direct, automated extraction ratio measurements. The organic and aqueous phases were detected by two independent diode array detectors controlled by computer.

In-line spectrophotometric detectors situated on a closed-loop have been used for continuous monitoring of the analytical signal. In the first arrangement, the accumulation of the extracted species is monitored directly, and a derivative can be formed inside the loop by addition of a suitable reagent. The other set-up involves measurement of the analyte after a preselected time delay, giving a typical flow injection analysis transient signal.
A waveguide long capillary cell made of Pyrex glass, wrapped with light reflective material and filled with sample solution has been applied as a spectrophotometric detector for the liquid-liquid extraction flow injection analysis determination of iodide.

An improvement to this system was made when a solvent was used which totally reflects the light inside the tube and propagates it through the capillary with a loss in intensity and applies it to liquid-liquid extraction-flow injection analysis for extremely sensitive determinations of copper with diethylthiocarbamate into carbon disulphide. A simple home made set-up of a detector based on a tungsten lamp (300W), an interference filter, and a capillary flow cell connected to the light source, provided a detection system and a liquid-liquid extraction-flow injection analysis system which was more useful.

Solid state, flow through detectors incorporating light-emitting diodes (LEDs) as sources of visible and UV radiation, and photodiode or phototransistor as a detector provide a simple, reliable and low-cost alternative to commercially available spectrophotometers. The LED's and photodiodes or phototransistors are relatively cheap, compact and commercially available components, the application of which minimizes sample dispersion within the detection system (Fig. 1.17).

Fig. 1.17: Flow through photometric detectors with a light emitting diode (LED) and phototransistor in (A) Z- and (B) on tube configurations.
In one of the most basic arrangements, the LED and phototransistor have been glued directly into a non-transparent detector body and the flowing stream comes into direct contact with the plastic surfaces of the solid state components, or both removable active components are located behind small glass focusing lenses. Another "on-tube" design uses non-intrusive solid-state components, and has the advantage of negligible sample dispersion with the flow cell which is made of Teflon or quartz (Fig. 1.17B). The arrangement could include a beam splitter for compensations in temperature change and ageing of the LED, and thus decrease long-term drift. A double beam arrangement uses separate LED-photodiode pairs to monitor the reference and sample streams.

A rapid "on-tube" photometric detection system controlled by computer is suitable for some applications of separator-less liquid-liquid extraction-flow injection analysis (Fig. 1.18).

Fig. 1.18: A flow injection extraction manifold with a coaxial segmenter and an "on-tube" rapid reading photometric detector controlled by computer for liquid-liquid extraction without phase separation (A) and a view of an "on-tube" flow through cell with transparent fluoropolymer, glass or quartz capillary (B).
The segmented flow is passed through the extraction measuring tube (Fig. 1.18B). The analytical signal is then measured by the detector and the analog signal fed to a computer and the data processed.

A similar detection system with a special glass capillary flow cell with optical fibres orientated perpendicularly to the main axis of the flow cell has been used for computer "phase separation" in liquid-liquid extraction-flow injection analysis and applied for the determination of pesticides in waste waters. A sophisticated "sorting" computer program allows the separation of analytical signals measured directly on the aqueous and organic segments, and subsequent determination of extractable species.

A laser excitation "on-tube" fluorimetric detector with a time resolution of 2ms, in which the laser beam of an argon laser was closely focused on the centre of a Teflon tube to eliminate the light scattering from the surface of the tube, has been used to study transient phenomena in the flowing stream and chelate formation between gallium(III) and lumogallion. Fluorescence was focused by a lens onto the entrance slit of a double monochromator and detected by a photomultiplier.

Chemiluminescence has an approximately fifty-fold higher sensitivity than that of fluorescence, however the precision is lower. It could be used in cases when no characteristic spectroscopic groups in molecular form are present, by using a simple home-made detection system with a spiral flow cell in front of a photomultiplier.

Flame AAS detectors are more often employed for the direct or indirect determination of analytes rather than graphite-furnace AAS instruments because of their simpler interconnection to the liquid-liquid extraction-flow injection analysis manifold. Introducing the controlled flow into a flow AAS detector by peristaltic pumping of the suitable phase stream improves the nebulizer performance, compared with aspiration controlled by the oxidant flow rate in the nebulizer.
An AAS detector with electrothermal atomization has been connected with a flow injection extraction manifold through an electronically controlled system of valves, and used for the determination of heavy metals\textsuperscript{87,88}, but the complexity of the system limits its practical uses.

1.5 Applications of liquid-liquid extraction-flow injection analysis.
It could reasonably be assumed that the development of a new liquid-liquid extraction-flow injection analysis procedure for the determination of an analyte would be a straightforward adaptation of an existing manual process to the liquid-liquid extraction-flow injection analysis manifold. However, nearly all the literature reports equilibrium data observed under equilibrium conditions. In addition, whenever masking of interferents is undertaken, manual procedures add masking reagents to the sample solution well in advance of analyte extraction, allowing complexation enough time to take place. In flowing systems, all the reactions take place more or less simultaneously, in less than ten seconds.

Therefore, liquid-liquid extraction in continuous flow analysis differs from that in manual procedures as equilibrium is rarely attained due to the limited sample residence time, the interfacial area between both phases, and the kinetics of the liquid-liquid extraction procedure.

Despite this, equilibrium data can serve as a good basis for the selection of the flow injection analysis experimental parameters, with the final choice made from experiments performed under exactly the same dynamic conditions as those for the analytical procedure.

1.5.1 Inorganic analytes.

Metals.
Alkali metals (Li, Na, K) in different types of water samples are determined by spectrophotometric or fluorimetric (Table 1.1) based on liquid-liquid extraction of an ion-pair as no suitable chelating reagent
exists. Coloured electroneutral ion-pairs formed between the metal ion, crown ether derivatives, and anionic chromophoric species, mostly azo or triphenylmethyl dyes, are normally extracted from alkaline solutions into a benzene/chlorobenzene mixture. Metal ions are determined using atomic absorption spectrometry and emission spectrometric or less selective spectrophotometric techniques (Table 1.1). Fluorimetric and other optical techniques are not used as frequently.

Table 1.1: Flow injection extraction determination of metal ions.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Matrix</th>
<th>Conditions</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li</td>
<td>Water</td>
<td>Dichloromethane</td>
<td>MAS</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Chloroform/crown ethers</td>
<td>MAS,ISE</td>
<td>90</td>
</tr>
<tr>
<td>K</td>
<td>River water</td>
<td>1,2 dichloroethane</td>
<td>Fl</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>River water</td>
<td>Benzene/chlorobenzene, A</td>
<td>MAS</td>
<td>91</td>
</tr>
<tr>
<td>Na,K</td>
<td>River/tap water</td>
<td>Benzene/chlorobenzene, B, Si-column</td>
<td>MAS</td>
<td>37</td>
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<tr>
<td>Ca</td>
<td>River water</td>
<td>Benzene/chlorobenzene, B</td>
<td>MAS</td>
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<tr>
<td>Cu</td>
<td>River water</td>
<td>MIBK, APDC</td>
<td>FAAS</td>
<td>34</td>
</tr>
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<td></td>
<td>Water</td>
<td>Dioxotetramine</td>
<td>ICP</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Standard, Water</td>
<td>Chloroform, DDC-Zn</td>
<td>FAAS</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>CS₂, DDC, of</td>
<td>MAS</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>MIBK, APDC</td>
<td>FAAS</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Chloroform, APDC</td>
<td>MAS</td>
<td>94</td>
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<tr>
<td>Zn</td>
<td>Biological environment</td>
<td>Chloroform, APDC</td>
<td>MAS</td>
<td>20</td>
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<td></td>
<td>Iron</td>
<td>MIBK, SCN</td>
<td>MAS</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>MIBK, SCN</td>
<td>FAAS</td>
<td>63</td>
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cont. Table 1.1
<table>
<thead>
<tr>
<th>Ion</th>
<th>Matrix</th>
<th>Conditions</th>
<th>Method</th>
<th>Reference</th>
</tr>
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<td>Cd, Pb</td>
<td>Water</td>
<td>Carbon tetrachloride, Dithizone, ec</td>
<td>MAS</td>
<td>96</td>
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<td>Carbon tetrachloride, Dithizone, PS</td>
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<td>Pb</td>
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<td>MIBK, APDC</td>
<td>FAAS</td>
<td>98</td>
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<td>Soil</td>
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<td>MIBK, I</td>
<td>MAS</td>
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<td>Fe, Cd, Ag</td>
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<td></td>
<td>AAS</td>
<td>100</td>
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<td>Co</td>
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<td>(Phen)_2P⁺</td>
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<td>MAS</td>
<td>38</td>
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<td>U</td>
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<td>MAS</td>
<td>55, 107</td>
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<td>Ga</td>
<td>Water</td>
<td>Isoamylalcohol, lumogallion</td>
<td>FL</td>
<td>73</td>
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<td>Mo</td>
<td>Plant</td>
<td>Isoamylalcohol, CNS and Fe</td>
<td>MAS</td>
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<td>Ti, Au</td>
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<td>MAS</td>
<td>102</td>
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<td>Rh</td>
<td></td>
<td>Crown ethers, Brilliant green</td>
<td>MAS</td>
<td>87</td>
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<td>Heavy metals</td>
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<td>MIBK, n-butylacetate</td>
<td>FAAS</td>
<td>70</td>
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<td>Water</td>
<td>MIBK, APDC</td>
<td>FAAS</td>
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<td>Blood serum</td>
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<td>ICP</td>
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<td>Water</td>
<td>Freon 113</td>
<td>GAAS</td>
<td>104</td>
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<tr>
<td>Water</td>
<td>Freon 113, APDC, DDDC, be</td>
<td>AAS</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Soil</td>
<td></td>
<td>FAAS</td>
<td>17</td>
<td></td>
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<tr>
<td>Polluted soil</td>
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<td>ICP</td>
<td>105</td>
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<tr>
<td>Water</td>
<td>Freon 113, APDC</td>
<td>GAAS</td>
<td>106</td>
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<tr>
<td>Seawater</td>
<td>Freon 113, APDC</td>
<td>GAAS</td>
<td>88</td>
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<tr>
<td>Water</td>
<td>Freon 113, APDC</td>
<td>GAAS</td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>

Inorganic ions.

Large inorganic anions, e.g., perchlorate, permanganate, nitrate etc., can be determined by direct spectrophotometric or indirect atomic absorption spectrometry techniques after liquid-liquid extraction into organic solvents in the form of ion-pairs with cationic organic dyes of the triphenylmethyl group, or with metal chelates of organic analytical reagents (Table 1.2).

Table 1.2: Flow injection extraction determination of large inorganic anions.

<table>
<thead>
<tr>
<th>Anion</th>
<th>Matrix</th>
<th>Conditions</th>
<th>Method</th>
<th>Reference</th>
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<tr>
<td>Perchlorate</td>
<td>Urine,</td>
<td>MIBK, A</td>
<td>indirect AAS</td>
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<tr>
<td></td>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perchlorate</td>
<td>Standard</td>
<td>Benzene, B</td>
<td>MAS</td>
<td>108</td>
</tr>
<tr>
<td>Nitrate/nitrite</td>
<td>Water</td>
<td>MIBK, C</td>
<td>indirect AAS</td>
<td>53</td>
</tr>
<tr>
<td>Nitrate/nitrite</td>
<td>Meat</td>
<td>MIBK, C</td>
<td>indirect AAS</td>
<td>109</td>
</tr>
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<td>Phosphate</td>
<td>Water</td>
<td>D, off d</td>
<td>Flame AAS</td>
<td>63</td>
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<td>Orthophosphate</td>
<td>Water</td>
<td>Benzene/</td>
<td>MAS</td>
<td>84</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<td>Permanganate</td>
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<td>Chloroform F</td>
<td>MAS</td>
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<td>Dichromate</td>
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<td>Chloroform G</td>
<td>111</td>
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<tr>
<td>Fluoride</td>
<td>Water</td>
<td>ICP</td>
<td></td>
<td>112</td>
</tr>
</tbody>
</table>

Key:  
A: [6-(methylpicolinealdehyde azine)-copper (I)]₂(CIO₄)₂;  
B: Brilliant green;  
C: bis-[2,9-dimethyl-1,10-phenanthrolinato copper (I)], neocuproine;  
D: n-butylacetate/molybdate;  
E: molybdophosphate and Malachite Green;  
F: ethylene-bis-(triphenylphosphonium) bromide;  
G: tetrachlorophosphate-bis-(triphenylphosphonium) bromide;  
off d: off line detection;  
MAS: spectrophotometry.  
AAS: atomic absorption spectrometry.
1.5.2 Organic analytes.

Surfactants.

Direct spectrophotometric methods for the determination of ionic surfactants are based on the formation of the stoichiometric, electroneutral ion pair of the composition e.g., $TR^+; T^+R^-$ etc., between a charged surfactant anion $T$ or cation $T^+$ and a cation or anion organic analytical reagent $R^+$ or $R^-$, respectively or with a suitable cationic metal chelate. The ion pairs are sparingly soluble in water but easily extractable into non-polar organic solvents e.g., chloroform, benzene and its chloro or alkyl-derivatives, or MIBK (Table 1.3).

The sensitivity of the determination is governed by the molar absorptivity of the chromophore, usually organic dye or metal chelate. Triphenylmethyl, thiazine and azo dyes are the most frequently used cationic dyes. The extractability of the ion pair of the cationic dye decreases in the order: ethyl violet, brilliant green, Hoffmann’s violet, crystal violet, malachite green, new fuchsin, methylene blue and pararosaniline, and the extraction efficiency of the solvents decreases in order: MIBK, 1,2, dichloroethane, chloroform, dichlorobenzene, monochlorobenzene and benzene$^{36,48,61,67,71,113}$.

Table 1.3: Flow injection extraction determination of surfactants.

<table>
<thead>
<tr>
<th>Type</th>
<th>Matrix</th>
<th>Conditions</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic</td>
<td>Water</td>
<td>Chloroform, MB</td>
<td>MAS</td>
<td>48</td>
</tr>
<tr>
<td>Sewage water</td>
<td>Toluene, EV</td>
<td>MAS, ns</td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>Water</td>
<td>Chloroform, MB</td>
<td>MAS, ns</td>
<td></td>
<td>41, 116</td>
</tr>
<tr>
<td>Waste waters</td>
<td>MIBK, A</td>
<td>indirect AAS</td>
<td></td>
<td>75</td>
</tr>
<tr>
<td>Water</td>
<td>Chloroform, MB</td>
<td>MAS, mc</td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>River water</td>
<td>Chloroform, B</td>
<td>MAS</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>River water</td>
<td>Chloroform, MB</td>
<td>MAS</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>Water</td>
<td>Chloroform, MB</td>
<td>MAS</td>
<td></td>
<td>67</td>
</tr>
</tbody>
</table>

cont/...
Table 1.3

<table>
<thead>
<tr>
<th>Type</th>
<th>Matrix</th>
<th>Conditions</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic</td>
<td>Water</td>
<td>Chloroform, MB</td>
<td>MAS, ip</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>Chloroform, Q</td>
<td>MAS</td>
<td>74</td>
</tr>
<tr>
<td>Cationic</td>
<td>Water, standard</td>
<td>Chloroform, OII</td>
<td>MAS</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>MIBK, C</td>
<td>indirect AAS</td>
<td>56</td>
</tr>
<tr>
<td>Nonionic</td>
<td>Water, Standard</td>
<td>1,2-dichloroethane, TBPEK</td>
<td>MAS</td>
<td>115</td>
</tr>
</tbody>
</table>

Note: MB: methylene blue; EV: Ethyl violet; OII: Orange II; A: Cu(I)-phenanthroline; B: 1-alkyl-4,4(4-diethylaminophenylazo) pyridinium chloride; C: terathiocyanatocobaltate (II); TBPEK: tetrabromophenolphthalein ethyl ester, potassium salt; Q: 4(4-N,N dimethylaminophenylazo)-2-methylquinoline; ns: nonsegmented; mc: micro-channel; ip: iterative procedure.

Drugs.
Spectrophotometric methods for the analysis of drugs in pharmaceutical preparations, tablets, sprays etc., (Table 1.4) are based on the principle of direct extraction of a coloured ion-pair formed between ionic drug species and suitable ionic colouring reagent, e.g., picrate and organic dyes containing a triphenylmethyl group. Chloroform is normally used as an extracting agent, however, 1,2-dichloroethane, cyclohexane and isoctanol also have an important function. Fluorimetric and chemiluminescence detection are useful for liquid-liquid extraction-flow injection analysis determination of drugs.

Other organic substances.
Non-ionic species can be separated and pre-concentrated by direct extraction into non-polar solvents using segmented or non-segmented liquid-liquid extraction-flow injection analysis. Such substances are determined as a sum of the extractable compound; pre-concentrated and partially separated by a multiple liquid-liquid extraction method including more than one extraction step involving one or several organic solvents; or are selectively determined by successive liquid chromatographic separation after a non-selective pre-concentration and separation step. Spectrophotometric or fluorimetric detection is generally preferred (Table 1.4).
Table 1.4: Flow injection extraction analysis of drugs and other types of organic substances.

<table>
<thead>
<tr>
<th>Compound(s)</th>
<th>Matrix</th>
<th>Conditions</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>Pharmaceutical</td>
<td>Chloroform</td>
<td>MAS, ec</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>tablets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coffee, tea, cocoa cola, aqueous</td>
<td>Chloroform</td>
<td>MAS</td>
<td>91,115</td>
</tr>
<tr>
<td>Codeine</td>
<td>Pharmaceutical</td>
<td>Chloroform</td>
<td>MAS</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>tablets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine (B&lt;sub&gt;12&lt;/sub&gt;)</td>
<td>Pharmaceuticals</td>
<td>Chloroform</td>
<td>MAS, Fl</td>
<td>54,118</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procyclidine hydrochloride</td>
<td>Pharmaceuticals</td>
<td>Chloroform</td>
<td>MAS, ec</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diphenhydramine and 8-chlorotheophylline</td>
<td>Dramamine tablets</td>
<td>Cyclohexane</td>
<td>MAS, bp</td>
<td>79</td>
</tr>
<tr>
<td>3,5-Dimethylphenol and p-toluidinium ion</td>
<td>pK&lt;sub&gt;a&lt;/sub&gt; determination</td>
<td></td>
<td>MAS</td>
<td>63</td>
</tr>
<tr>
<td>Steroid sulphate and bile acids</td>
<td>Biological material</td>
<td>Chloroethane, bromothymol blue</td>
<td>Fl, Cl</td>
<td>84</td>
</tr>
<tr>
<td>Tetrodoline</td>
<td>Blood serum</td>
<td>n-heptane</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>Enalapril</td>
<td>Pharmaceuticals</td>
<td>1,2-dichlorothene, luteigen</td>
<td>MAS</td>
<td>66</td>
</tr>
<tr>
<td>Phenylephrine hydrochloride and phenyramine</td>
<td>Nasal spray</td>
<td>Chloroform</td>
<td>MAS</td>
<td>50</td>
</tr>
<tr>
<td>Berberine and benzethonium</td>
<td>Drugs</td>
<td>TBPEK</td>
<td>MAS</td>
<td>119</td>
</tr>
<tr>
<td>Quaternary ammonium</td>
<td>Standards</td>
<td>Chloroform</td>
<td>MAS</td>
<td>30</td>
</tr>
<tr>
<td>Polyaromatic compounds</td>
<td>Shale oil</td>
<td>DMSO/pentane</td>
<td>Fl/hplc</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DMSO/water</td>
<td>Fl/hplc</td>
<td>21,120</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>Vegetable oils</td>
<td>Toluene</td>
<td></td>
<td>121,122</td>
</tr>
<tr>
<td>Halocarbons</td>
<td>Seawater</td>
<td></td>
<td>GC/MAS</td>
<td>123</td>
</tr>
<tr>
<td>Amines</td>
<td>Aqueous/aqueous</td>
<td>MAS/hplc</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Bittering compounds</td>
<td>Beer</td>
<td>Isotane</td>
<td>MAS</td>
<td>60</td>
</tr>
<tr>
<td>Pesticides</td>
<td>n-heptane</td>
<td>MAS-uv/hplc</td>
<td></td>
<td>124</td>
</tr>
<tr>
<td>Partition coefficient</td>
<td>Standards</td>
<td>Chloroform</td>
<td>MAS</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: DMSO: Dimethylsulphoxide; MAS: spectrophotometry; TBPEK: tetrabromophenolphthalein ethyl ester, potassium salt; ec: determination of extraction constants; Cl: chemiluminescence; Fl: fluorescence; bp: simultaneous monitoring of both phases; GC: gas chromatography; hplc: high performance liquid chromatography.
CHAPTER TWO

FLUORESCENCE
2.1 Introduction.
One of the most advantageous features of fluorescence is the intrinsic sensitivity, with detection limits being one to three orders of magnitude smaller than those encountered in absorption spectroscopy. Typical detection limits range from a few parts per thousand to less than parts per million. Another advantage of fluorescence methods are the linear concentration ranges, which are often significantly greater than those observed with absorption methods. Finally, the selectivity of fluorescence procedures is at a minimum as good as or in some cases even better than that of absorption methods due to the relatively limited number of chemical systems that produce fluorescence.

2.2 Theory of fluorescence.
Fluorescent behaviour takes place in simple, as well as complex gaseous, liquid and solid chemical systems. The simplest type of fluorescence is that observed with dilute atomic vapours; e.g., the 3s electrons of vaporised sodium atoms can be excited to the 3p state by absorption of radiation of wavelengths 598.6 and 589nm, after approximately 10ns, the electrons return to the ground state, and in doing so emit radiation of the same two wavelengths in all directions; this type of fluorescence in which the absorbed radiation is re-emitted without any change is known as resonance radiation or resonance fluorescence.

Molecular species also show fluorescence occasionally. However, more often, molecular fluorescence occurs as bands of radiation that are centred at wavelengths that are longer than the resonance lines. This shift toward longer wavelengths is called the Stokes Shift.

2.2.1 Molecular orbital considerations.
The characteristics of fluorescent spectra can be rationalized by means of simple molecular orbital considerations. Absorbing species containing π (π), σ (σ) and non-bonding (n) electrons include organic
molecules and ions as well as a number of inorganic anions. This discussion will concentrate on the former, although the latter will be considered briefly.

The electrons that contribute to excitation by an organic molecule are:

1. those that participate directly in bond formation between atoms and are therefore associated with more than one atom;

2. non-bonding or unshared outer electrons that are largely localized about such atoms as oxygen, the halogens, sulphur and nitrogen.

Covalent bonding takes place due to the electrons forming the bond, moving in the field about two atomic centres in such a manner as to minimize the repulsive coulombic forces between these centres. The non-localised fields between atoms that are occupied by bonding electrons are called molecular orbitals and can be considered to result from the overlap of atomic orbitals. When two atomic orbitals combine, either a low-energy bonding molecular orbital or a high energy antibonding molecular orbital results. The electrons of a molecule occupy the former in the ground state.

The molecular orbitals associated with single bonds in organic molecules are designated as sigma (\(\sigma\)) bonds, and the corresponding electrons are \(\sigma\) electrons. The distribution of charge density of a sigma orbital (Fig. 2.1) is rotationally symmetric around the axis of the bond. Here, the average negative charge density arising from the motion of the two electrons around the two positive nuclei is indicated by the degree of shading.
The double bond in an organic molecule contains two sorts of molecular orbitals: a sigma (σ) orbital corresponding to one pair of the bonding electrons and a pi (π) orbital associated with the other pair. Pi orbitals are formed by the parallel overlap of atomic p orbitals. Their charge distribution is characterised by a nodal plane, which is a region of low charge density, along the axis of the bond and a maximum density in the regions above and below the plane (Figs. 2.1a and 2.1b) also shown (Figs. 2.1c and 2.1d) are the charge density distributions for antibonding sigma and pi orbitals; these orbitals are designated by σ' and π'.

In addition to the σ and π electrons, many organic compounds contain non-bonding electrons. These unshared electrons are designated by the symbol O in Fig. 2.2.

![Electron distribution in sigma-bonding and non-bonding orbitals](image)

Fig. 2.1: Electron distribution in sigma-bonding (a) and non-bonding (c) and pi-bonding (b) and non-bonding (d) molecular orbitals.

The energies for the various types of molecular orbitals differ (Fig. 2.3) significantly. Generally, the energy level of a non-bonding electron lies between those of the bonding and the non-bonding pi and sigma orbitals.
Electronic transitions among certain energy levels can be brought about by the absorption of radiation. Four types are possible: $\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$, $n \rightarrow \pi^*$, and $\pi \rightarrow \pi^*$ (Fig. 2.3).

$\sigma \rightarrow \sigma^*$ transitions.
This is where an electron in a bonding $\sigma$ orbital of a molecule is excited to the corresponding antibonding orbital by the absorption of radiation. The molecule is then described as being in the $\sigma, \sigma^*$ excited state. Relative to other possible transitions, the energy required to induce a $\sigma \rightarrow \sigma^*$ transition is large (Fig. 2.3), corresponding to radiant frequencies in the vacuum ultraviolet region.

Excitation maxima due to $\sigma \rightarrow \sigma^*$ transitions are never observed in the ordinary, accessible ultraviolet/near infra red region and therefore do not warrant further discussion.

![Electronic molecular energy levels depicting different types of transition.](image)

$n \rightarrow \sigma^*$ transitions.
Saturated compounds containing atoms with unshared electron pairs (non-bonding electrons) are capable of $n \rightarrow \sigma^*$ transitions. Generally, these
transitions require less energy than the $\sigma \rightarrow \sigma^*$ type and can be brought about by radiation in the region of between 150 and 250nm. Excitation maxima for the formation of the $n,\sigma^*$ state tend to shift to shorter wavelengths in the presence of polar solvents such as water or ethanol.

**$n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions.**

Most applications of excitation to organic compounds are based upon transitions for $n$ or $\pi$ electrons to the $\pi^*$ excited state because the energies required for these processes bring the excitation peaks into an experimentally convenient spectral region (200-900nm). Both transitions require the presence of an unsaturated functional group to provide $\pi$ orbitals.

One characteristic difference between the two types of transition is the effect exerted by the solvent on the wavelength of the peaks. Peaks associated with the $n \rightarrow \pi^*$ transitions are generally shifted to shorter wavelengths i.e., a hypsochromic or blue shift with increasing polarity of the solvent. In the majority of cases, the reverse trend i.e., a bathochromic or red shift, is observed for $\pi \rightarrow \pi^*$ transitions. The hypsochromic effect arises from the increased solvation of the un-bonded electron pair, which lowers the energy of the $n$ orbital. The most significant effects of this kind i.e., blue shifts of 30nm or more, are seen with polar hydrolytic solvents, such as water or alcohols, in which hydrogen-bond formation between solvent protons and the non-bonded electron pair is extensive. The energy of the $n$ orbital is lowered by an amount approximately equal to the energy of the hydrogen bond. When a $n \rightarrow \pi^*$ transition takes place, however, the remaining single $n$ electron cannot maintain the hydrogen bond; therefore the energy of the $n,\pi^*$ excited state is not affected by this type of solvent interaction. A blue shift, approximately corresponding to the energy of the hydrogen bond, is therefore seen.
A second solvent effect that influences both $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions leads to a bathochromic shift with increased polarity. This effect is small, usually less than 5nm, and is therefore insignificant in the $n \rightarrow \pi^*$ transitions by the hypsochromic effect. With the bathochromic shift, attractive polarization forces between the solvent and the absorber (i.e., species to be excited) tend to lower the energy levels of both the unexcited and the excited states. The effect on the excited state is greater, and as a result the energy difference becomes smaller with increased polarity; small bathochromic shifts result.

2.2.2 Singlet and triplet state considerations.

Although the characteristics of the fluorescent spectra can be rationalized by molecular orbital considerations (section 2.2.1), an understanding of the phenomena needs a review of electron spin and singlet and triplet excited states.

**Electron spin.**

To explain the behaviour of atoms and molecules in strong magnetic fields, it is necessary to assume that each electron in an atom or molecule has associated with it a magnetic field that arises from a spinning motion of the electron around its axis. It also has to be assumed that only two quantized spin states can exist, with the direction of rotation and therefore the associated magnetic field being opposite for the two. In a molecular orbital containing two electrons, the spins of the two are opposed as a consequence of the Pauli exclusion principle which states no two electrons in one atom can have all four quantum numbers the same, i.e., each atomic orbital can contain no more than two electrons of opposite spin. Under these circumstances, the spins are termed as being paired. As a result of spin pairing, most molecules have no net magnetic field and are termed diamagnetic, i.e., they are repelled by permanent magnetic fields. In contrast, free radicals, which contain an unpaired electron, have a magnetic moment and as a result are attracted into a magnetic field and are termed paramagnetic.
Singlet/triplet excited states.
When one pair of electrons of a molecule is excited to a higher energy level, a singlet or triplet state is permitted (Fig. 2.4).

![Diagram of electron spin in various states](image)

**Fig. 2.4: Diagrammatic representation of electron spin in various states.**

*NB: The excited triplet state is less energetic than the corresponding singlet state.*

In the excited singlet state, the spin of the promoted electron is still paired with the ground state electron; in the triplet state, the spin of the two electrons become unpaired and parallel.

The properties of a molecule in the excited triplet state differ significantly from those of the excited singlet state. However, more importantly, is the fact that a singlet-triplet transition, or the reverse, which also involves a change in electronic state, is a significantly less probable event than the corresponding singlet-singlet transition. For this reason, the average lifetime of an excited triplet state may range from $10^{-4}$ to several seconds, as compared with an average lifetime of $10^{-6}$ to $10^{-8}$ seconds for an excited singlet state. In addition, radiation induced excitation of a ground-state molecule to an excited triplet state does not take place readily, and absorption peaks due to this process are several orders of magnitude less intense than the analogous singlet-singlet transition.
2.2.3 Energy level considerations for photoluminescent molecules.

A partial energy level diagram for a typical photoluminescent molecule is shown in Fig. 2.5. The lowest heavy horizontal line represents the ground state energy of the molecule, which is normally a singlet state and is labelled $S_0$. At room temperature, this state represents the energies of essentially all of the molecules in a solution.

![Energy level diagram for a photoluminescent system](image)

Fig. 2.5: Partial energy diagram for a photoluminescent system.

The upper heavier lines are energy levels for the ground vibrational states of three excited electronic states. The two lines on the left represent the first ($S_1$) and second ($S_2$) electronic singlet states. The one on the right ($T_1$) represents the energy of the first excited triplet state. The energy of the first triplet state is lower than the energy of the corresponding singlet state. A number of vibrational energy levels are associated with each of the four electronic states, as shown by the lighter horizontal lines.
As shown in Fig. 2.5, excitation of a molecule can be brought about by absorption of two bands of radiation, one centred about the wavelength \( \lambda_1(S_0\rightarrow S_1) \) and the second around the shorter wavelength \( \lambda_2(S_0\rightarrow S_2) \). It must be noted that the excitation process results in conversion of the molecule to any of the several excited vibrational states. In addition, direct excitation to the triplet state is not depicted because this transition does not occur to any significant extent as this process involves a change in multiplicity, an event which has a low probability of taking place, this is often termed forbidden.

2.2.4 Deactivation processes.

An excited molecule can return to its ground state by a combination of several mechanistic steps. As depicted by the straight vertical arrows in Fig. 2.5, two of these steps fluorescence and phosphorescence, involve the release of a photon of radiation. The other deactivation steps, indicated by wavy arrows, are radiationless processes. The favoured route to the ground state is the one that minimizes the lifetime of the excited state. Therefore, if deactivation by fluorescence is rapid with respect to the radiationless process, fluorescence is favoured. On the other hand if the radiationless deactivation has a more favourable rate constant, fluorescence is either absent or less intense.

The photoluminescent phenomena is limited to a relatively small number of systems incorporating structural and environmental features that cause the rate of radiationless deactivation processes to be slowed to a point where the emission reaction can compete kinetically. Information concerning emission processes is sufficiently complete to permit a quantitative accounting of their rates. For deactivation processes only qualitative statements about rates and mechanisms can be made. Nevertheless, the interpretation of photoluminescence requires consideration of these other routes.
Rates of absorption and emission.
The rate at which a photon of radiation is absorbed is vast, the process can take place in $10^{14}$ to $10^{15}$ seconds. Fluorescent emission, however, occurs at significantly slower rate. An inverse relationship exists between the lifetime of the excited state and the molar absorptivity of the absorption peak corresponding to the excitation process. Therefore, for molar absorptivities in the $10^3$ to $10^5$ range, lifetimes of the excited state are $10^{-7}$ to $10^{-9}$ seconds. For weakly absorbing systems, where the probability of the transition process is less, lifetimes may be as long as $10^{-6}$ to $10^{-5}$ seconds.

Vibrational relaxation.
As can be seen from Fig. 2.5, a molecule may be promoted to any of several vibrational levels during the electronic excitation processes. In solution, however, the excess vibrational energy is immediately lost as a result of collisions between the molecules of the excited species and those of the solvent, the result is an energy transfer and a minuscule rise in temperature of the solvent. This relaxation process is so efficient that the average lifetime of a vibrationally excited molecule is $10^{-12}$ seconds or less, this period is significantly shorter than the average lifetime of an electronically excited state. As a result, fluorescence from a solution always involves a transition from the lowest vibrational level of an excited state to any one of the vibrational levels of the ground state (Fig. 2.5).

A consequence of the efficiency of vibrational relaxation is that the fluorescence band for a given electronic transition is displaced toward lower frequencies or longer wavelengths from the absorption band, i.e., Stokes shift, overlap takes place only for the resonance peak involving transitions between the lowest vibrational level of the ground state and the corresponding level of the excited state.
Internal conversion.
The term internal conversion is employed to describe intermolecular processes by which a molecule passes to a lower energy electronic state without emission of radiation. These processes are neither well defined nor well understood, but it is apparent that they are often highly efficient, since relatively few compounds exhibit fluorescence.

Internal conversion appears to be particularly efficient when two electronic energy levels are sufficiently close for the existence of an overlap in vibrational energy levels. This situation is depicted for the two singlet states in Fig. 2.5. At the overlaps depicted, the potential energies of the two excited states are identical; this equality apparently allows an efficient transition. Internal conversion through overlapping vibrational levels is generally more likely than the loss of energy by fluorescence from a higher excited state. Therefore, excitation by radiation $\lambda_2$ (Fig. 2.5) would normally produce fluorescence of wavelength $\lambda_3$ to the exclusion of a band that would result from a transition between $S_2$ and $S_0$. In this case, the excited molecule proceeds from the higher electronic state to the lowest vibrational state of the lower electronic excited state via a series of vibrational relaxations, an internal conversion, and then further relaxations. Under these circumstances, the fluorescence would be of $\lambda_3$ only, regardless of whether radiation of wavelength $\lambda_1$ or $\lambda_2$ was responsible for the excitation.

The mechanisms of the internal conversion process $S_1 \rightarrow S_0$ (Fig. 2.5) are not well understood. The vibrational levels of the ground state may overlap those of the first excited electronic state, under such conditions, deactivation will occur rapidly by the mechanism outlined above. This situation is apparent with, for example, aliphatic compounds and accounts for the fact that these species rarely fluoresce; deactivation by energy transfer through overlapping vibrational levels takes place so rapidly that fluorescence does not have time to occur.
Internal conversion may also result in the phenomena of predissociation. In this case, the electron moves from a higher electronic state to an upper vibrational level of a lower electronic state in which the vibrational energy is large enough to cause rupture of a bond. In a large molecule, there is an increased probability for the existence of bonds with strengths less than the electronic excitation energy of the chromophores. Rupture of these bonds can take place as a result of absorption by the chromophore followed by internal conversion of the electronic energy to vibrational energy associated with the weak bond.

Predissociation should be differentiated from dissociation, in which the absorbed radiation excites the electron of a chromophore directly to a sufficiently high vibrational level to cause breakage of the chromophoric bond: no internal conversion is involved. Dissociation processes also compete with the fluorescence process.

External conversion.

Deactivation of an excited electronic state may involve interaction and energy transfer between the excited molecule and the solvent or other solutes. These processes are called external conversion. Evidence for external conversions includes the marked effect upon fluorescent intensity exerted by the solvent; furthermore, those conditions that tend to reduce the number of collisions between particles e.g., low temperature and high viscosity, generally lead to enhanced fluorescence. The details of external conversion processes are not well understood.

Radiationless transitions to the ground state from the lowest excited singlet and triplet states (Fig. 2.5) probably involve external conversions, as well as internal conversions.
**Intersystem crossing.**

Intersystem crossing is a process in which the spin of an excited electron is reversed, and a change in multiplicity of the molecule results. As with internal conversion, the probability of this transition is enhanced if the vibrational levels of the two states overlap. The singlet-triplet transition shown in Fig. 2.5 is an example: in this case the lowest singlet vibrational state overlaps one of the upper triplet vibrational levels and a change in spin is thus more probable.

### 2.3 Variables that affect fluorescence.

Both molecular structure and chemical environment are influential in determining whether a substance will or will not fluoresce; these facts also determine the intensity of emission when fluorescence does occur. The effects of some of these variables is discussed below.

#### 2.3.1 Quantum yield.

The quantum yield, or quantum efficiency, for a fluorescent process is simply the ratio of the number of molecules that fluoresce to the total number of excited molecules. For a highly fluorescent molecule such as fluorescein, the quantum efficiency under some conditions approaches one.

From a consideration of Fig. 2.5 and the discussion of deactivation processes, it is evident that the fluorescent quantum yield $\phi$ for a compound must be determined by the relative rate constants $k_x$ for the processes by which the lowest excited singlet state is deactivated, primarily fluorescence ($k_f$), internal conversion ($k_{ic}$), external conversion ($k_{ec}$), intersystem crossing ($k_i$), predissociation ($k_{pd}$), and dissociation ($k_d$). These relationships may be expressed as,
\[ \phi = \frac{k_f}{(k_f + k_i + k_{ee} + k_{ie} + k_{pd} + k_o)} \]  \hspace{1cm} (2.1)

where the \( k \) terms are the respective rate constants for several processes in equation 2.1. Equation 2.1 allows a qualitative interpretation of many of the structural and environmental factors that influence fluorescent intensity. Evidently, these variables that lead to high values for the fluorescence rate constant \( k_f \) and low values for the other \( k \) terms enhance fluorescence. The magnitude of \( k_o \), the predissociation constant \( k_{pd} \), and the dissociation rate constant \( k_o \) are mainly dependent upon chemical structure, the remaining constants are strongly influenced by environment and to a lesser extent by structure.

2.3.2 Transition types in fluorescence.

It must be noted that fluorescence rarely results from absorption of ultraviolet radiation of wavelengths lower than 250nm as this type of radiation is not energetic enough to cause deactivation of the excited states by predissociation or dissociation. As a result fluorescence due to \( \sigma \rightarrow \sigma^* \) transitions is rarely seen; such emission is confined to the less energetic \( \pi \rightarrow \pi^* \) and \( \pi \rightarrow n \) processes.

As stated previously, an electronically excited molecule ordinarily returns to its lowest state by a series of rapid vibrational relaxations and internal conversions that produce no emission of radiation. Therefore, any fluorescence observed most commonly arises for a transition from the first excited electronic state to one of the ground state vibrational levels. For the majority of fluorescent compounds, radiation is produced by a transition involving either the \( n,\pi^* \) or the \( \pi,\pi^* \) excited state, depending upon which is less energetic.
2.3.3 Quantum efficiency and transition time.

It has been determined experimentally that fluorescent behaviour is more commonly observed in compounds in which the lowest energy excited state is of a $\pi,\pi^*$ type than those with a lowest energy $n,\pi^*$ state i.e., the quantum efficiency is greater for $\pi-\pi^*$ transitions.

The greater quantum efficiency associated with the $\pi,\pi^*$ state can be explained in two ways. First, the molar absorptivity of a $\pi-\pi^*$ transition is ordinarily one hundred to one thousand fold greater than for a $n-\pi^*$ process and this quantity represents a measure of transition probability in either direction. Therefore, the inherent lifetime associated with a $\pi-\pi^*$ transition is shorter e.g., $10^{-7}$ to $10^{-9}$ seconds compared to $10^{-5}$ to $10^{-7}$ seconds for a $n,\pi^*$ state and $k_i$ in equation 2.1 is larger.

It is also thought that the rate constant for intersystem crossing $k_i$ is smaller for $\pi,\pi^*$ excited states because the energy difference between the singlet-triplet states is larger, i.e., more energy is needed to unpair the electrons of the $\pi,\pi^*$ excited state. Consequently, overlap of triplet vibrational levels with those of the singlet state is reduced, and the probability of an intersystem crossing is smaller.

In short, fluorescence is more commonly associated with $\pi,\pi^*$ states than with $n,\pi^*$ states as the former possesses a shorter average lifetime ($k_i$ is larger) and also as the deactivation processes that compete with fluorescence are less likely to take place.

2.3.4 Fluorescence and structure.

The most intense and useful fluorescence is found in compounds containing aromatic functional groups with low energy $\pi-\pi^*$ transition levels. Compounds containing aliphatic and alicyclic carbonyl structures may also exhibit fluorescence, however the range of these is much smaller compared to the range in the aromatic systems.
Most unsubstituted aromatic hydrocarbons fluoresce in solution, the quantum efficiency usually increases with the number of rings and their degree of condensation. The simple heterocyclics, e.g., pyridine, furan, thiophene and pyrrole, do not show fluorescent behaviour; however fused-ring structures ordinarily do. With nitrogen heterocyclics, the lowest-energy electronic transition is thought to involve a $\pi^* \rightarrow \pi$ system that rapidly converts to the triplet state and prevents fluorescence. Fusion of benzene rings to a heterocyclic nucleus, results in an increase in the molar absorbptivity of the absorption peak. The lifetime of an excited state is shorter in such structures; fluorescence is therefore observed for compounds such as quinoline, isoquinoline, and indole.

Substitution on the benzene ring\textsuperscript{123} causes shifts in the wavelength of absorption maxima and corresponding changes in the fluorescence peaks. In addition, substitution often affects the fluorescence efficiency, some of these effects are shown by the data for benzene derivatives in Table 2.1.

The influence of halogen substitution is striking; the decrease in fluorescence with increasing atomic number of the halogen is thought to be due partly to the heavy atom effect, which increases the probability of intersystem crossing to the triplet state. Predissociation is thought to play an important role in iodobenzene and in nitro derivatives as well; these compounds have easily ruptured bonds that can absorb the excitation energy following internal conversion.

Substitution of a carboxylic acid or carbonyl group on an aromatic ring generally inhibits fluorescence. In these compounds, the energy of the $n,\pi^*$ system is less than in the $\pi,\pi^*$ system, as the fluorescent yield from the former type of system is ordinarily low.
Table 2.1: Effect of substitution on the fluorescence of benzene.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>$\lambda_{\text{excitation}}$, nm</th>
<th>$\lambda_{\text{emission}}$, nm</th>
<th>Relative Emission Intensity of Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>C₆H₆</td>
<td>270-310</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Toluene</td>
<td>C₆H₅CH₃</td>
<td>270-320</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>C₆H₅C₆H₇</td>
<td>270-320</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Fluorobenzene</td>
<td>C₆H₅F</td>
<td>270-320</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>C₆H₅Cl</td>
<td>275-345</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Bromobenzene</td>
<td>C₆H₅Br</td>
<td>290-380</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Iodobenzene</td>
<td>C₆H₅I</td>
<td>No fluorescence</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Phenol</td>
<td>C₆H₅OH</td>
<td>285-365</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Phenolate ion</td>
<td>C₆H₅O⁻</td>
<td>310-400</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Anisole</td>
<td>C₆H₅OCH₃</td>
<td>285-345</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Aniline</td>
<td>C₆H₅NH₂⁺</td>
<td>310-405</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Anilinium ion</td>
<td>C₆H₅NH₂⁺</td>
<td>No fluorescence</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>C₆H₅COOH</td>
<td>310-390</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>C₆H₅CN</td>
<td>280-360</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>C₆H₅NO₂</td>
<td>No fluorescence</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

NB: The measurements are taken in ethanol.

Effect of structural rigidity.

It is found empirically that fluorescence is favoured in molecules that possess rigid structures, e.g., the quantum efficiencies for fluorene and biphenyl (Fig. 2.6) are nearly 1.0 and 0.2 respectively, under similar conditions of measurement.
Fig. 2.6: Structure of (A) fluorene and (B) biphenyl.

The difference in behaviour appears to be largely as a result of the increased rigidity enabled by the bridging methylene group in fluorene. In addition, enhanced emission frequently results when fluorene is adsorbed on a solid surface, the added rigidity provided by the solid surface may account for the observed effect.

The influence of rigidity has also been given as a reason for the increase in fluorescence of certain organic chelating agents when they are complexed with a metal ion, e.g., the fluorescent intensity of 8-hydroxyquinoline is much less than that of the zinc complex:  

$$\text{structure below}$$

Lack of rigidity in a molecule probably causes an enhanced internal conversion rate and a consequent increase in the likelihood for radiationless deactivation. One part of a non-rigid molecule can undergo low-frequency vibrations with respect to other parts; such motions account for some energy loss.
2.3.5 Temperature and solvent effects.

The quantum efficiency of fluorescence by most molecules decreases with increasing temperature due to the increased frequency of collisions at elevated temperatures, thereby increasing the probability for deactivation by external conversion. A decrease in solvent viscosity also increases the likelihood of external conversion and leads to the same result.

The polarity of the solvent also has an important influence. The energy for \( \pi \rightarrow \pi^* \) transitions is often increased in polar solvents, while that for \( \pi \rightarrow \pi^* \) transitions experiences an opposite effect. Such shifts may occasionally be large enough to lower the energy of the \( \pi \rightarrow \pi^* \) processes below that of the \( n \rightarrow \pi^* \) transition; enhanced fluorescence results.

The fluorescence of a molecule is decreased by solvents containing heavy atoms or other solutes with such atoms in their structure e.g., carbon tetrachloride, ethyl iodide. The effect is similar to that which occurs when heavy atoms are substituted into fluorescent compounds; orbital spin interaction results in an increase in the rate of triplet formation and a corresponding decrease in fluorescence.

2.3.6 Effect of pH on fluorescence.

The fluorescence of an aromatic compound with acidic or basic ring substituents is usually pH dependent. Both the wavelength and the emission intensity will probably be different for the ionized and non-ionized forms of the compounds. The data for phenol and aniline shown in Table 2.1 illustrates this effect. The changes in emission of compounds of this type arise from the differing number of resonance species that are associated with the acidic and basic forms of the molecules, e.g., aniline has several resonance forms:
while the anilinium ion has one:

The additional resonance forms lead to a more stable first excited state, fluorescence in the ultraviolet region is the consequence.

The fluorescent behaviour of certain compounds as a function of pH has been used for the detection of end points in acid-base titrations, e.g., fluorescence of the phenolic form of 1-naphthol-4-sulphonic acid is not detectable visually as the fluorescence occurs in the ultraviolet region. When the compound is converted to the phenolate ion by the addition of base, however, the emission peak shifts to visible wavelengths where it can be readily observed. It is of interest that this change takes place at a different pH than would be predicted from the acid dissociation constant for the compound. The reason for this discrepancy is that the acid dissociation constant of the excited molecule differs from that for the same species in its ground state. Changes in acid or base dissociation constants with excitation are common and are occasionally as large as four or five orders of magnitude. It is obvious from these observations that analytical procedures based on fluorescence frequently require close control of pH.
2.3.7 Effect of dissolved oxygen.

The presence of dissolved oxygen often reduces the emission intensity of a fluorescent solution. This effect may be the result of a photochemically induced oxidation of the fluorescent species. More commonly, the quenching takes place as a result of the paramagnetic properties of molecular oxygen, which promotes intersystem crossing and conversion of excited molecules to the triplet state. Other paramagnetic species also tend to quench fluorescence.

2.3.8 Effect of concentration on fluorescent intensity.

The power of fluorescent radiation, $F$, is proportional to the radiant power of the excitation beam that is absorbed by the system, i.e.,

$$F = k'(P_o - P)$$  \hspace{1cm} (2.2)

where $P_o$ is the power of the beam incident upon the solution and $P$ is the power after traversing a length, $b$, of the medium. The constant $k'$ depends upon the quantum efficiency of the process. In order, to relate $F$ to the concentration, $c$, of the fluorescing particle, Beer's law has the form;

$$\left(\frac{P}{P_o}\right) = 10^{-\varepsilon bc}$$  \hspace{1cm} (2.3)

where $\varepsilon$ is the molar absorptivity of the fluorescent molecules and $\varepsilon bc$ is the absorbance, $A$. By substitution of equation 2.3 into 2.2, the following is obtained,

$$F = k'P_o(1 - 10^{-\varepsilon bc})$$  \hspace{1cm} (2.4)

The exponential term in equation 2.4 can be expanded as a series to,

$$F = k'P_o \left[2.3\varepsilon - \frac{(2.3\varepsilon bc)^2}{2!} + \frac{(2.3\varepsilon bc)^3}{3!}\right]$$  \hspace{1cm} (2.5)

provided $\varepsilon bc = A < 0.05$, all of the subsequent terms become negligible with respect to the first, i.e., equation 2.5 becomes,

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or at constant $P_o$.

\[ F = k c \quad 2.7 \]

Therefore, a plot of the fluorescent power of a solution versus concentration of the emitting species should be linear at low concentrations, $c$. When $c$ becomes large enough to give an absorbance of 0.05 the higher order terms in equation 2.5 become important and linearity no longer exists; $F$ then lies below an extrapolation of the straight line plot.

Two other factors, also attribute for negative deviations from linearity at high concentration, these are self-quenching and self-absorption. The former is the result of collisions between excited molecules. Radiationless transfer of energy occurs, probably in a similar manner to the transfer of solvent molecules that takes place in an external conversion. Self-quenching can be expected to increase with concentration due to the increased probability of collisions taking place.

Self absorption takes place when the wavelength of emission overlaps an absorption peak, fluorescence is then decreased as the emitted beam traverses the solution.

The effects of these phenomena are such that a plot relating fluorescent power to concentration may exhibit a maximum.

### 2.4 Emission and excitation spectra.

An excitation spectrum is obtained by measuring luminescence intensity at a fixed wavelength while the excitation is varied. Emission (fluorescence) spectra involve excitation at a fixed wavelength while recording the emission intensity as a function of wavelength. Often the emission and excitation
spectra are approximate mirror images of each other due to the vibrational energy differences for the ground and excited electronic states being approximately the same (Fig. 2.5).

2.5 Instruments for measuring fluorescence.

The various components of instruments for measuring fluorescence are similar to those found in the ultraviolet/visible spectrophotometers or photometers. A typical configuration of the components in a fluorimeter is shown in Fig. 2.7.

Nearly all fluorescence instruments use double-beam optics as shown (Fig. 2.7) in order to compensate for fluctuations in the power of the source. The sample beam first passes through an excitation filter or a monochromator, which transmits radiation that will excite fluorescence but excludes or limits the emitted radiation. Fluorescent radiation is emitted from the sample in all directions but is most conveniently observed perpendicularly to the excitation beam, at other angles, increased scattering from the solution and the cell walls may cause large errors in the measurement of intensity. The emitted radiation reaches a photoelectric detector after passing through a second filter or monochromator that isolates a fluorescent peak for measurement.

The reference beam passes through an attenuator that reduces its power to approximately that of the fluorescent radiation (usually by a factor of 100 or more). The signals from the reference and sample phototubes are then fed into a difference amplifier whose output is displayed by a meter or recorder. Many fluorimeters are of the null type, this state being achieved by optical or electrical attenuators.
Fig. 2.7: Components of a fluorimeter and spectrofluorimeter.

2.5.1 Components of fluorimeters and spectrofluorimeters.

Sources
For most applications, a more intense source is needed than the tungsten usually employed for the measurement of absorption. As shown in equation 2.6, the magnitude of the output signal, and thus the sensitivity, is directly proportional to the source power $P_0$. A mercury or xenon arc lamp is normally used.

The xenon arc lamp produces intense radiation by the passage of current through an atmosphere of xenon. In some instruments, regularly spaced flashes are obtained by discharging a capacitor through the lamp; higher intensities are realized in this way. In addition, the output of the phototubes is then a.c., which can be readily amplified and processed.

Mercury arc lamps produce an intense line spectrum at a number of wavelengths. As fluorescent behaviour can be induced in most fluorescing compounds by a variety of wavelengths, at least one of the mercury lines is usually suitable.
A recent important development has been the use of various types of lasers as excitation sources for fluorescence measurements, e.g., a tunable dye laser as the primary source, monochromatic radiation between 360nm and 650nm is produced. Such a device eliminates the need for an excitation monochromator.

**Filters and monochromators.**
Both interference and absorption filters have been used in fluorimeters. Most spectrofluorimeters are equipped with grating monochromators.

**Detectors.**
A typical fluorescent signal is of low intensity, large amplification factors are therefore needed for its measurement. Photomultiplier tubes have come into widespread use as detectors in sensitive fluorescence instruments. Diode array detectors have also been proposed.

**Cells and cell compartments.**
Both cylindrical and rectangular cells of glass, silica, or acrylic have been used to make fluorescence measurements. Care must be taken in the design of the cell compartment to reduce the amount of scattered radiation reaching the detector. Baffles are often introduced into the compartment for this purpose.

### 2.6 Long wavelength fluorescence.
There has in recent years, been a major expansion in the area of molecular fluorescence. Advances in instrumentation and data handling methods and the synthesis of new fluorescent labels and probe molecules, have combined to produce a wide range of new applications, many of them novel and most of them in the areas of biological and environmental chemistry. The advantages of fluorescence methods in these areas include their exceptional sensitivity, the ease with which different sample handling methods can be used to combine
fluorimetry with other techniques and the sensitivity of molecular fluorescence phenomena to environmental effects, which is the basis of several elegant techniques. Nevertheless, most of these fluorescence methods use the ultraviolet/visible region of the spectrum, i.e., 250-500nm, and this presents a number of serious practical difficulties.

2.6.1 Problems associated with ultraviolet-visible fluorescence.

The major disadvantage of the ultraviolet/visible fluorescence methods is the extent to which they are liable to unwanted background signals. It is known that fluorimetry is in principle very sensitive as it aims to detect small emission signals against a zero or near-zero background; this gives it an enormous advantage over absorption spectrometry, which in trace analysis involves detecting a small difference between two large light signals. It follows that if the background signal in fluorimetry is not zero or nearly so, the method loses its main advantage. In practice, background signals can be of two types:

Rayleigh scattering.

Rayleigh scattering (by the solvent in dilute solution fluorimetry), in which the scattered photons have the same wavelength as the incident photons, is present in all fluorescence experiments. It is minimised by the use of filters or monochromators in the fluorimeter; by the right angled optics; and where possible by the use of fluorophores which have large Stokes shifts. Rayleigh scattered light is strongly polarised, so the insertion of an appropriate polariser may also help to reduce its intensity. However, its principal feature is that, for a given sample, its intensity varies as the inverse fourth power of the incident light wavelength. In instruments which use a xenon arc lamp or a pulsed xenon lamp as the light source, the combination of this wavelength dependence and that of the light source intensity means that the Rayleigh signal is largest when an excitation wavelength of ~350nm is used, unfortunately this wavelength region is one where many common
fluorophores are excited, so the background effect can be serious despite all precautions.

**Raman scattering.**

Raman scattering is a much weaker effect than the Rayleigh effect, but can be just as disadvantageous. Again it is solvent Raman scattering that is normally observed, therefore the effects are predictable if the solvent Raman spectrum is available. Most fluorescence experiments in biological and environmental chemistry utilise aqueous solution and the major Raman band of water is at $\sim3400\text{cm}^{-1}$. Therefore, when an excitation wavelength of 350nm is used the water Raman signal is at 397.5nm, where it can clearly interfere with genuine fluorescence signals. When peptides and proteins containing tyrosine are excited in aqueous solution at the amino acids absorption maxima of 278nm, the water Raman band occurs at 307nm, almost exactly coincident with the tyrosine fluorescence emission maxima.

### 2.6.2 Why long wavelength fluorescence?!

In most applications, fluorophores are used to improve the sensitivity of other methods,\textsuperscript{26} e.g., chromatographic separation techniques, immunoanalytical or enzymatic methods. The fluorophores chosen, in the main, emit in the visible spectral region. One of the most widely used fluorophores is fluorescein. Although fluorescein has a high quantum yield, its absorption and emission maxima are in the spectral region where interference is likely to occur, although less than in the ultraviolet or the short-wavelength visible spectral region. The practical applicability of any fluorescent labelling method depends upon both its intrinsic sensitivity and on the presence of interfering fluorophores. Fluorophores with longer absorption and emission wavelengths, especially long wavelength chromophores, offer advantages with regard to interference as only a few groups of compounds are able to absorb and fluoresce in this spectral region. These advantages are clearly shown in Fig. 2.8.
2.6.3 Long wavelength fluorophores.

The major requirement for fluorophores are high quantum yield, large Stokes shift, excitation maxima accessible to inexpensive light sources, chemical and photochemical stability, low susceptibility to fluorescence quenchers, and reactive functional groups in the molecule. There is an enormous number of fluorophores available for use in the ultraviolet/visible spectral region. These labels have most of the desired characteristics; however, their relatively short wave absorption and fluorescence maxima ensure that the background fluorescence of the matrix is large.

There are fewer groups of compounds that have their absorption and emission maxima in the long wavelength spectral region as opposed to the ultraviolet and visible regions. Long wavelength absorption and emission fluorophores need to have extensive conjugation in the molecule. Two major families of dyes are known to have long wavelength absorption maxima, the cyanine and phthalocyanine dye groups. In addition, to these two groups, certain phycobiliproteins can exhibit fluorescence in the long wavelength spectral region. Phycobiliproteins have very large molar absorptivities and good quantum yields; however, their large molecular weights (≈100,000) disadvantages their application. Also, there are artificial phycobilisome molecules.
known to fluoresce in the long wavelength spectral region$^{133}$. A wide range of fluorophores are known in the cyanine group, $\sim 3000$ have been reported. They have large molar absorptivities ($\sim 200,000$) and reasonably good quantum yields ($\sim 0.8$). A tabular representation (Table 2.2) shows how the spectral characteristics of cyanine dyes are determined by their chemical structure.

Table 2.2: Spectral characteristics from the chemical structure of cyanine dyes.

<table>
<thead>
<tr>
<th>Length of chain (R)</th>
<th>$\lambda_{\text{absorbance}}, \text{nm}$</th>
<th>$\lambda_{\text{fluorescence}}, \text{nm}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Cyanine Dye 1" /></td>
<td>1</td>
<td>490</td>
</tr>
<tr>
<td>2</td>
<td>585</td>
<td>620</td>
</tr>
<tr>
<td>3</td>
<td>680</td>
<td>740</td>
</tr>
<tr>
<td><img src="image2" alt="Cyanine Dye 2" /></td>
<td>1</td>
<td>550</td>
</tr>
<tr>
<td>2</td>
<td>650</td>
<td>690</td>
</tr>
<tr>
<td>3</td>
<td>750</td>
<td>790</td>
</tr>
<tr>
<td><img src="image3" alt="Cyanine Dye 3" /></td>
<td>1</td>
<td>560</td>
</tr>
<tr>
<td>2</td>
<td>660</td>
<td>690</td>
</tr>
<tr>
<td>3</td>
<td>785</td>
<td>810</td>
</tr>
</tbody>
</table>

Another important characteristic of long wavelength fluorophores is their susceptibility to different fluorescence quenching processes. Fluorescent quenchers increase the probability of the excited fluorophore returning to the ground state via non-radiative processes thereby reducing fluorescence efficiency of the fluorophore. In order to prevent reduced efficiency, either the concentration of potential quenchers must be
decreased or labels that are less susceptible to quenching need to be used. Fortunately, the short fluorescence lifetime of long wavelength fluorophores decreases the probability of non-radiative quenching processes.

A systematic study of the influence of quenchers on the fluorescence of long wavelength fluorophores has been described\(^\text{134}\). Different ionic and non-ionic quenchers were used to classify the fluorophores. The information obtained is important to the design of appropriate labelling systems. Several of the fluorophores were found not to be particularly sensitive to quenching processes. This indicates the structure is an important criterion for choosing the appropriate label. Typical Stern-Volmer constants for long wavelength fluorophores are so low that no significant quenching can be expected in most bioanalytical applications\(^\text{134}\).

2.6.4 Application of long wavelength fluorophores.

Long wavelength fluorophores may be classified into two groups, covalently and non-covalently attached labels. It is possible to attach fluorophore molecules using non-covalent means, e.g., non-specific binding to molecules, adsorption, or electrostatic forces. In several cases the much weaker non-covalent binding mechanism provides suitable stability during the analysis. Covalent attachments, although preferred, have the disadvantage of requiring fluorophores containing the appropriate functional groups. This need may produce limitations and the fluorophores are not commercially available. The fluorophore label should have the appropriate moieties that can react with functional groups on the analyte to form covalent bonds between the analyte molecule and the fluorophore\(^\text{127}\). Due to the lack of commercial availability of long wavelength fluorophores, several research groups have exploited the application of non-covalently bound labels\(^\text{128,135,136}\).
Non-covalent fluorophore labels.

There are several examples of applications of non-covalently bound long wavelength fluorophores. Competitive binding of indocyanine green (ICG) to human and bovine serum albumin was studied by Kamisaka et al\textsuperscript{135}. One of the earliest procedures that used this binding for analytical applications was shown by Sauda et al\textsuperscript{136}. These researchers recognized that indocyanine green, which binds strongly to serum albumins, could be used as a long wavelength fluorophore. Protein in human serum was labelled with indocyanine green, separated on a gel filtration column, and detected by semiconductor laser fluorimetry. Picomolar detection limits were reported\textsuperscript{128,136}. Although there are serious problems with the non-covalently labelled fluorophores, i.e., they tend to dissociate on the chromatographic column during separation, the advantages of long wavelength labels are in evidence.

Application of non-covalently and covalently bound labels may need careful evaluation. The binding of long wavelength fluorophores to albumins or globulins is a good example. Four different cyanine dyes were evaluated to determine binding to serum proteins\textsuperscript{137}. Although these four fluorophores (Fig. 2.9) are structurally similar, significant differences were observed as to how the spectral properties of each fluorophore changed upon binding to large molecules.

Pronounced differences were observed in their spectra, indicating that specific binding may occur between protein and certain fluorophores. The DTTCI complexed with albumins, especially human serum albumin, and a significant decrease in the absorption and fluorescence was observed, whereas the other three did not\textsuperscript{137}. The results suggest that the presence of sulphur heteroatoms and their distance from each other are the determining factors in the observed phenomena. Non-specific binding may also be present in covalently attached fluorophores. This secondary labelling effect should be minimized, especially in biological systems.

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3,3'-diethylthiadicarbocyanine iodide (DTDCI)

3,3'-diethylthiatricarbocyanine iodide (DTTCI)

3,3'-diethyloxadicarbocyanine iodide (DODCI)

3,3'-diethyloxatricarbocyanine iodide (DOTCI)

Fig. 2.9: Chemical structures of four long wavelength fluorophores similar in structure.
Non-specific binding to serum proteins, however, can be helpful in determining protein characteristics. The long wavelength fluorophores often exhibit significant spectral changes. This effect may or may not be desirable, depending on the application. Certainly, changes in the micro-environment should be avoided during the analytical procedure. However, this phenomena may be advantageous if the micro-environment of the fluorophore needs to be monitored simultaneously. An example of this application is the monitoring of hydrophobicity using long wavelength fluorophores, acting as non-covalent labels, it is possible to monitor the hydrophobicity in micro-environments simultaneously. An approach to monitoring micro-hydrophobicity by fluorescence lifetime of long wavelength fluorophores was reported by Imasaka et al.Ionic forces between two oppositely charged molecules can be used for labelling purposes. Polymethine cyanine fluorophores, which are ionic molecules, can be used for determining ionic analytes, such as surfactants. It was also found that the selection of solvent can have a significant effect on the overall performance of the method. The use of long wavelength fluorophores can reduce sample preparation time because of less interference in this spectral region.

Another application of a non-covalent long wavelength fluorophore is in vivo cell tracing with cyanine dyes. In this method viable cells are labelled and their life spans are followed using fluorimetry. In vivo cellular tracking of labelled blood cells may be used for diagnosing tumours and other diseases. The application of visible fluorophores is severely limited for bio-molecule determinations because of strong interferences.

Carbocyanine dyes are useful as molecular probes in membranes. Low interference of the relatively long absorption and emission wavelengths
of cyanine dyes is useful when the technique is coupled with fluorescence microscopy. Significant enhancement was observed in their fluorescence intensity as they bind to synthetic bilayers. Menzel et al. have used cyanine dyes to study electrical damage in insulators by attaching fluorophores to damaged surfaces.

Fluorophore labelling may be combined with other techniques such as fluorescence microscopy to study several important parameters e.g., surface organization, physical state, dynamics and cell functions, of cell surfaces. Most fluorophores that can react with proteins are suitable for such work. Relatively little research has been carried out on the application of long wavelength absorbing fluorophores for cell labelling. Hydrophobic carbocyanine dyes have been used frequently. An effective method for increasing fluorescence intensity in the long wavelength region has been described by Oi et al., who used phycobiliproteins, which may be directly conjugated to antibodies, biotin or avidin for labelling conjugated antibodies.

Covalent long wavelength fluorophores.
For bio-molecule labelling and for analytes containing primary amino functional groups, isothiocyanate derivatives of fluorophores are the most suitable labels because they form stable thioureas. Amino derivatives, which are precursors to isothiocyanate-substituted dyes, can also be used to conjugate the fluorophore to thiol groups on proteins. A reagent which uses this chemistry is N-succinimidyl-3-(2-pyridyldithio) propionate, which has been widely used in immunochemistry.

Fluorescein and rhodamine isothiocyanates are some of the most widely used visible absorbing fluorophores. Mujumdar et al., reported isothiocyanate derivatives of cyanine dyes for medical applications using two-colour cytometry. Lymphocyte types were identified using fluorescein and cyanine fluorescence signals, and distinct separation of
cell types was achieved. Some labels exhibit significant absorption around 780nm allowing laser diode excitation with fluorescence detection to be used.

The long wavelength fluorophores containing the iodoacetamide reactive group have been described for labelling protein sulphydryl residues. Iodoacetamide derivatives of cyanine and merocyanine fluorophores were synthesized for covalently labelling proteins and other biomolecules. These dyes have been found to have high molar absorptivities and moderate quantum yields.

2.6.5 Difficulties associated with applications of long wavelength fluorophores.

Fluorescence in the long wavelength region requires extensive conjugation in the molecule, which in turn creates disadvantages. The fluorophores are necessarily large, hydrophobic molecules, often with molecular weights close to 1000. This is not really a disadvantage if large biomolecules are labelled. The extensive conjugation increases its instability, which can result in photobleaching or shortened shelf life of the fluorophore. This instability may be reduced with appropriate dye design, e.g., using phthalocyanines or incorporating the polymethine chains into cyclic structures.
CHAPTER THREE

MATERIALS, INSTRUMENTS AND GENERAL PROCEDURES
3.1 Instrumentation and equipment.

3.1.1 Instrumentation.

Fluorescence measurements were carried out using a Perkin Elmer LS50 luminescence spectrofluorimeter fitted with a R928 photomultiplier tube to enable spectra to be taken at the longer wavelengths involved in this project. The LS50 was interfaced to an Epson AX3 personal computer fitted with the Fluorescence Data Manager (FLDM) software package which controlled all aspects of the spectrofluorimeter.

Absorbance measurements were made using either the Uvikon 810 ultraviolet-visible spectrophotometer connected to a Kontron chart recorder, or the Unicam 8700 series ultraviolet-visible spectrophotometer connected to a Unicam colour plotter. The diluent buffer or organic solvent used for extraction was used as the reference.

All pH measurements were made with a calibrated Corning 140 pH meter, fitted with a saturated calomel electrode.

3.1.2 Equipment.

Peristaltic pump.

A Gilson miniplus 3 peristaltic pump was employed, in the flow injection manifold, to propel the aqueous and organic phases. The pump was capable of propelling eight channels, and the pump head consisted of ten rollers.

Injection valve.

The injection valve (20μl), used in the flow extraction manifold was a low pressure Teflon rotary valve, Rheodyne 5020. All parts of the valve in contact with phases employed in the manifold were constructed of Teflon, the remaining parts of the valve were either stainless steel or polypropylene. The valve had six ports, two ports formed the sample injection loop, one was a waste port for excess sample, one for the introduction of the sample, and the remaining two ports for the entry and exit of the carrier stream i.e., buffer, through the injection valve.
Cells.

All fluorescence measurements, using the manual extraction procedure, were made using 1cm path length Hellma silica fluorescence cells.

For all the absorbance measurements 1cm path length, matched, Hellma cells were used.

The organic phase being continually passed through the cell destroyed the seals of the commercially available flow cells and as a result a flow through cell with no seals was designed, and manufactured at Loughborough University. The flow cell consisted of two parts, the first was a right-angled silica tube (Fig. 3.1A) with a screw type fitting at one end. The second part was a blackened aluminium block with slots of varying dimensions (Fig. 3.1B).

![Fig. 3.1: Schematic representation of (A) the silica tube and (B) the blackened aluminium block.](image)

The silica tube (Fig. 3.1A) was held in place by the aluminium block in the cuvette holder of the LS50. The aluminium block (Fig. 3.1B) had three slots of dimensions 24mm length and 3mm width and one blind side, one of the faces with a slot also had an additional slot 9mm long.
and 6mm wide (Fig. 3.1B) this additional slot was present to accommodate the curved portion of the silica tube and also to position the cell in a reproducible manner each time it was removed from the cuvette holder. A side view of the complete flow cell is given in Fig. 3.2. Once the cell was positioned an illuminated volume of 18μl was achieved with the excitation slit of the LS50 set at 5nm.

![Fig. 3.2: Schematic representation of the flow cell used for this project.](image)

Phase separator.
A design based on those proposed by Fossey and Cantwell¹³,⁶²,⁷⁶ and Imasaka et. al.,¹³⁹,¹⁴⁰ was constructed from 25% glass filled PTFE at Loughborough University. More details are given in section 3.3.2.

Miscellaneous.
PTFE transmission tubing of internal diameter 0.8mm and flow injection connectors were obtained from Omnifit. Glass Y-shaped segmenters were made at Loughborough University, and PTFE tape, (this was to be used as the membrane), was obtained from a local DIY store.
3.2 Materials and preparation of solutions.

3.2.1 Materials.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Supplier and description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium cromoglycate (Intal)</td>
<td>Fisons Research.</td>
</tr>
<tr>
<td>Warfarin</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>DL Propranolol</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Nedocromil</td>
<td>Fisons Research</td>
</tr>
<tr>
<td>Barbitone</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Sulphadiazine</td>
<td>British Drug Houses</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Sulphameth oxazole</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Amitryptilline</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td><strong>Fluorophores</strong></td>
<td></td>
</tr>
<tr>
<td>1,1',3,3',3',3',3'-hexamethyldotricarbocyanine perchlorate (HIDTCP) Laser grade</td>
<td>Eastman Kodak</td>
</tr>
<tr>
<td>Methylene Blue B.P.</td>
<td>Boots</td>
</tr>
<tr>
<td>Rhodamine 800</td>
<td>Lambda Physik</td>
</tr>
<tr>
<td>3,3',-diethyloxacarbocyanine iodide (DODCI) Laser grade</td>
<td>Eastman Kodak</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Fluka Chemicals</td>
</tr>
<tr>
<td>Buffer materials</td>
<td>Source</td>
</tr>
<tr>
<td>-------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>2-(N-morpholino)ethane sulphonic acid (mes)</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Ethylenedioxyethylene dinitrotetraacetic acid (EGTA)</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>British Drug Houses</td>
</tr>
<tr>
<td>Citric acid</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>Potassium hydrogen carbonate</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>Potassium hydrogen phthalate</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>Disodium tetraborate (Borax)</td>
<td>East Anglia Chemicals</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Fisons Scientific</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Miscellaneous</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>Fisons Scientific</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Carless</td>
</tr>
<tr>
<td>Human serum albumin crystallized and lyophilized, fatty acid and globulin free.</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Dimethylmaleonate</td>
<td>Lancaster Chemicals</td>
</tr>
<tr>
<td>Diethylacetoacetate</td>
<td>Lancaster Chemicals</td>
</tr>
</tbody>
</table>

Deionized, triply distilled water from a Liqui-pure Modulab system.
3.2.2 Preparation of buffer solutions.

A number of buffers were used.

**Citrate Buffer pH 2.5**
For a litre of citrate buffer.
29.26g of sodium chloride (0.5M)
and 21.0g of Citric acid (0.1M)
were made up in deionized, triply distilled water.
The buffer was adjusted to a pH of 2.5 with either 5M sodium hydroxide
or 5M hydrochloric acid and then stored at 4°C.

**Acetate Buffer pH 4**
For a litre of acetate buffer.
11.6ml of glacial acetic acid (0.2M)
and 8.203g for sodium acetate (0.1M)
were made up in deionized, triply distilled water.
The buffer was adjusted to a pH of 4 with either 5M sodium hydroxide
or 5M hydrochloric acid and then stored at 4°C.

**Mes Buffer pH 6.9**
For a litre of mes buffer.
19.52g of mes (0.1M)
0.38g of EGTA (1mM)
and 0.203g of magnesium chloride (1mM).
was made up in deionized, triply distilled water.
The buffer was adjusted to a pH of 6.9 with either 5M sodium hydroxide
or 5M hydrochloric acid and then stored at 4°C.

**Borate Buffer pH 10**
For a litre of borate buffer.
8g of sodium hydroxide (0.2M)
and 9.534g of Borax (0.02M).
was made up in deionized, triply distilled water.
The buffer was adjusted to a pH of 10 with either 5M sodium hydroxide
or 5M hydrochloric acid and then stored at 4°C.
Chloride buffer pH 2.5
For chloride buffer:
250ml of 0.2M potassium chloride (3.728g) was added to 100ml of 0.2M hydrochloric acid.
The buffer was adjusted to a pH of 2.5 with either 5M sodium hydroxide or 5M hydrochloric acid and then stored at 4°C.

Phthalate buffer pH 4
For phthalate buffer:
A 0.05M solution of potassium hydrogen phthalate was prepared.
The buffer was stored at 4°C.

Phosphate buffer pH 6.9
For phosphate buffer:
250ml of 0.1M potassium hydrogen phosphate (3.4g) was added to 73ml of 0.2M sodium hydroxide (0.584g).
The buffer was adjusted to a pH of 6.9 with either 5M sodium hydroxide or 5M hydrochloric acid and then stored at 4°C.

Carbonate buffer pH 10
For carbonate buffer:
300ml of 0.025M sodium hydrogen carbonate (0.63g) was added to 300ml of 0.025M sodium carbonate (0.79g).
The buffer was adjusted to a pH of 10 with either 5M sodium hydroxide or 5M hydrochloric acid and then stored at 4°C.

3.2.3 Preparation of drug solutions.
The drugs were prepared as stock solutions in neutral buffer and stored at 4°C for no longer than seven days. However flufenamic acid, sulphaemethaxazole, sulphadiazine and warfarin were not soluble in the neutral buffer and required a slightly alkaline media which was obtained by adding a drop of 0.88M ammonia to the stock solution.
Diazepam and digitoxin were prepared in methanol as they were completely insoluble in aqueous media. It must be noted that all subsequent dilutions were carried out in the appropriate buffer for the system being considered.

3.2.4 Fluorophore preparation.

All the labels were insoluble in neutral buffer, and therefore the stock solutions were prepared in methanol. As with the solutions of the drugs, subsequent dilutions were made using the appropriate buffer for the system being considered. The stock solutions were prepared daily and stored at 4°C.

NB: All the solutions were allowed to reach room temperature before use.

3.3 Experimental.

3.3.1 Optimisation of manual procedure.

All the flow injection experimentation was carried out only after preliminary work considering both fluorescence and absorbance data using the manual extraction procedure.

As the phase separator dictated that the organic phase be of greater density than the aqueous, in order for the extraction to proceed, the choice of solvent was limited. Solvents commonly employed in manual liquid-liquid extraction were considered. Carbon tetrachloride, chloroform and dichloromethane were considered and as with all chlorinated solvents a disadvantage of quenching in the fluorescence measurements was observed. Table 3.1 shows the fluorescence intensities observed when shaking 5ml of the same concentration of Rhodamine 800 (12nM, for structure see section 4.3) with 5ml of the solvent for 30s.
Table 3.1: Fluorescence intensity observed after a manual extraction procedure for a 12nM solution of Rhodamine 800.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt;</th>
<th>Absorbance</th>
<th>λ&lt;sub&gt;ex&lt;/sub&gt;</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt;</th>
<th>Emission Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane</td>
<td>685</td>
<td>0.755</td>
<td>685</td>
<td>704</td>
<td>76</td>
</tr>
<tr>
<td>Chloroform</td>
<td>685</td>
<td>0.588</td>
<td>685</td>
<td>704</td>
<td>66</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

*Key: abs: absorbance; ex: excitation; em: emission.*

As a consequence of the values observed in Table 3.1, carbon tetrachloride was no longer considered due to the extent of the quenching. Of the remaining solvents, dichloromethane gave as expected slightly less quenching than chloroform. However, difficulties arose when using dichloromethane in a flow injection manifold. Constant air-bubble formation meant that it would be difficult practically to measure the peak area of the signal resulting from an on-line liquid-liquid extraction; i.e., a measurement of peak area is less difficult with a peak shape shown in Fig. 3.3A than with a peak shape shown in Fig. 3.3B.

![Fig. 3.3](image_url): Typical flow extraction peaks, (A) with no air bubbles reaching the flow cell and (B) with air bubbles reaching the flow cell.
It was decided therefore that chloroform be used as the difficulties of air-bubble formation were not as extreme when it was considered in a flow extraction manifold.

Ordinarily, the time for which the aqueous and organic phase are shaken together is crucial. This is important as equilibrium needs to be attained before separation of the two phases can take place. However, the same is not true of on-line liquid-liquid extraction (see chapter one, section 1.4.3), and due to this fact investigation of the time for which the two phases should be shaken before which equilibrium were attained was not undertaken.

Preliminary experiments were carried out by shaking 5ml of the:
1. dye only, i.e. blank;
2. dye plus the drug in a 1 dye:10 drug ratio;
3. dye plus the drug in a 1 dye:1 drug ratio;
4. dye plus the drug in a 10 dye:1 drug ratio.
with 5ml of chloroform for 30s in a 50ml separating funnel. It must be noted that the concentration of the dye was kept constant throughout the experiment. The absorbance, fluorescence excitation and emission spectra were recorded. A full calibration, i.e., considering a wide range of drug concentrations, keeping the dye concentration constant, was considered if significant differences in the emission signal were seen when compared to the signal of the blank. The absorbance and fluorescence excitation measurements were only considered for the preliminary experiments. It must be noted that blank values appeared to be very high for some measurements, this was due to systematic errors.

3.3.2 Optimisation of the flow injection liquid-liquid extraction.

The flow injection manifold is shown schematically in Fig. 3.4. The components are as described in chapter one, section 1.4. All the optimisation studies to be discussed were carried out using methylene blue and warfarin using the conditions shown in Table 3.2.
Fig. 3.4: Schematic diagram of the flow injection liquid-liquid extraction manifold.

Table 3.2: Conditions for optimisation of the flow injection extraction manifold.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of methylene blue</td>
<td>20μM</td>
</tr>
<tr>
<td>Concentration of warfarin</td>
<td>20mM</td>
</tr>
<tr>
<td>Slits (excitation and emission)</td>
<td>5nm</td>
</tr>
<tr>
<td>Aqueous phase</td>
<td>pH 10 (Borate)</td>
</tr>
<tr>
<td>Excitation wavelength</td>
<td>655nm</td>
</tr>
<tr>
<td>Emission wavelength</td>
<td>664nm</td>
</tr>
</tbody>
</table>

The transport unit was a peristaltic pump (described in section 3.1). The pump tubing for the aqueous phases was constructed of PVC, and that for the chloroform and recirculated waste was made of Viton®; the diameters of the tubing used are given in Table 3.3.

Table 3.3: Inner diameter of tubing used.

<table>
<thead>
<tr>
<th>Material</th>
<th>Inner diameter</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic</td>
<td>1.5mm</td>
<td>Viton®</td>
</tr>
<tr>
<td>Recirculated waste</td>
<td>0.7mm</td>
<td>Viton®</td>
</tr>
<tr>
<td>Dye</td>
<td>1.0mm</td>
<td>polyvinyl chloride</td>
</tr>
<tr>
<td>Buffer</td>
<td>1.0mm</td>
<td>polyvinyl chloride</td>
</tr>
<tr>
<td>Transmission tubing</td>
<td>0.8mm</td>
<td>Teflon</td>
</tr>
</tbody>
</table>

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The diameters of the tubing was kept constant and the speed of the 
pump altered by means of a switch at the front of the pump. The data 
for the optimisation is given in Table 3.4.

Table 3.4: Flow rates of the individual streams and peak areas for the 
conditions listed in Table 3.2.

<table>
<thead>
<tr>
<th>Line</th>
<th>Mean flow rates ml/min</th>
<th>Mean peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dye</td>
<td>Buffer</td>
</tr>
<tr>
<td>1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>8</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>9</td>
<td>2.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

N.B.: All the measurements were made in triplicate however only the mean values for the 
flow rates and peak area are given.

The most obvious choice, for the pump speed, from the data given in 
Table 3.4, would be when the flow rates are as detailed in 'line 1' of 
Table 3.4, as the greater peak area is observed. However a number of 
other factors have to be taken into consideration; the first is the time 
taken for the analysis. The time of analysis (Fig. 3.5) would be far 
greater for the flow rates equivalent to those in 'line 1' of Table 3.4 
(peak area=7925) compared those detailed in 'line 9' of Table 3.4 
(peak area=1296). The second consideration is one of dispersion; the 
slower the pump speed the greater the dispersion. In flow analysis the 
ideal peak shape should be as shown in Fig 3.3(A).
Fig. 3.5: Peaks observed at differing pump speeds.

Key: 1-line 1 of Table 3.4', 2-line 2 of Table 3.4', 3-line 3 of Table 3.4', 4-line 4 of Table 3.4', 5-line 5 of Table 3.4', 6-line 6 of Table 3.4', 7-line 7 of Table 3.4', 8-line 8 of Table 3.4', 9-line 9 of Table 3.4'.
A compromise needs to be considered between peak area, peak shape and speed of analysis. In this case, flow rates detailed in 'line 5' of Table 3.4 were chosen, and these were used for all further experimentation.

The solvent segmenters were of the Y-type (Fig. 3.6) and constructed of glass at Loughborough University. Omnifit Teflon T-pieces were considered, but abandoned due to the problems encountered with respect to back pressure.

![Diagram of segmenter](image)

**Fig. 3.6:** Schematic diagram of the segmenter used.

The mixing coil was constructed from 0.8mm inner diameter (i.d.) Teflon transmission tubing, and the length of this was varied and the peak area recorded. The configuration of the coil was dependant upon the length to be considered, smaller coils i.e., less than 50cm, were knotted, longer coils were wound round a plastic pipe. Table 3.5 gives the peak areas observed as the length of the mixing coil was varied.

**Table 3.5:** Effect of varying mixing coil length.

<table>
<thead>
<tr>
<th>Mixing coil length/m</th>
<th>Mean Peak Area/ arbitrary units</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>976</td>
</tr>
<tr>
<td>0.2</td>
<td>1380</td>
</tr>
<tr>
<td>0.5</td>
<td>1225</td>
</tr>
<tr>
<td>1.0</td>
<td>1164</td>
</tr>
<tr>
<td>1.5</td>
<td>1308</td>
</tr>
</tbody>
</table>
A mixing coil length of 0.2m was taken as being optimum and this length was used in all subsequent work.

Extraction coil length was found, as expected, to increase the dispersion in direct proportion to length (Fig. 3.7).

Fig. 3.7: A diagrammatic representation to show how peak shape varies with extraction coil length.

The coil was constructed, from 0.8mm i.d., teflon transmission tubing, the required length was wound round a length of plastic pipe as with the mixing coils. Table 3.6 gives the peak area recorded for differing extraction coil lengths.
Table 3.6: Effect of varying extraction coil length.

<table>
<thead>
<tr>
<th>Extraction coil length/m</th>
<th>Peak area/ arbitrary units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>1878</td>
</tr>
<tr>
<td>1.0</td>
<td>2333</td>
</tr>
<tr>
<td>1.5</td>
<td>1776</td>
</tr>
<tr>
<td>2.0</td>
<td>2147</td>
</tr>
<tr>
<td>2.5</td>
<td>1511</td>
</tr>
<tr>
<td>5.0</td>
<td>1489</td>
</tr>
</tbody>
</table>

As seen in Fig. 3.7 dispersion and time of analysis increased as the length of the extraction coil increased. Although the extraction coil of 1m gives the greatest peak area, the peak areas of the individual measurements are not reproducible. An extraction coil length of 0.5m was chosen as it gives the greatest reproducibility with respect to peak area and also has a more rapid time of analysis.

A restrictor coil, which consisted of teflon transmission tubing (internal diameter 0.8mm), with 'a loosely tied knot in it', was used to regulate the overpressure across the teflon membrane. This coil produced a slight back pressure forcing the organic phase through the membrane.

The phase separator (Fig. 3.8) as stated earlier in section 3.1 is the most important component of the flow injection liquid-liquid extraction manifold. The design of the separator used for this project is a combination of designs suggested by Fossey and Cantwell\textsuperscript{13,62,76} and Imasaka et al.,\textsuperscript{139,140}. The material from which the phase separator was constructed was 25% glass filled PTFE. This was an improvement on an earlier design which was constructed of PTFE. The disadvantage of the previous separator was that the separator became deformed. Glass filled PTFE did not show this. The ports of the separator were designed so the
CHAPTER FOUR

USE OF LONG WAVELENGTH FLUOROPHORES WITH ON-LINE LIQUID-LIQUID EXTRACTION
4.1 Introduction.
This chapter will describe which long wavelength fluorophores were used, and how the fluorescence emission signals were affected by liquid-liquid extraction in the presence of drugs for varying values of pH. Differing buffer systems were considered as pH exerts more influence on fluorescence than on absorbance as a result of the rate of protolytic dissociation being greater than the time taken for fluorescence decay to occur.

4.2 Methylene Blue.

Methylene blue (structure shown) has, to-date, been used primarily as an indicator and also in liquid-liquid extraction with spectrophotometric detection. As explained in Chapter 3, section 3.3.1, preliminary work involved shaking 5ml of an aqueous solution of the fluorophore and drug with 5ml of chloroform. The absorbance and fluorescence (excitation and emission) spectra of the decanted organic layer after shaking were measured and the values observed used to determine whether or not to pursue the study of the particular drug/fluorophore combination.

4.2.1 Methylene blue using an aqueous phase of pH 2.5 (citrate buffer).
Fig. 4.1 shows the fluorescence (emission and excitation) spectra for the organic phase after shaking an aqueous solution of methylene blue with chloroform. The data obtained for the preliminary study to determine the extractability of methylene blue (pH 2.5, citrate buffer) and named drugs is shown in Table 4.1. However, the preliminary data for further fluorophore/drug combinations will be represented as a bar chart of the
fluorescence emission data, but it must be noted that the absorbance and fluorescence excitation measurements were still made.

Fig. 4.1: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when methylene blue (pH 2.5, citrate buffer) was shaken with chloroform using manual liquid-liquid extraction.

Table 4.1: Preliminary data obtained to determine the extractability of methylene blue and named drugs (pH 2.5, citrate buffer) using manual liquid-liquid extraction.

<table>
<thead>
<tr>
<th>Drug</th>
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### Table 4.1

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NB: Concentration of methylene blue = 2μM; Slits of spectrofluorimeter 5nm. *absorbance units.

From the data given in Table 4.1, changes in the measured emission signal for the differing ratios of the drug to methylene blue compared to the blank were observed when sodium cromoglycate, DL propranolol and warfarin were present with methylene blue (pH 113
2.5, citrate buffer), suggesting that a calibration may be possible. These drugs were considered for a calibration using manual liquid-liquid extraction (Fig. 4.2). There does not appear to be a trend, as one would expect for a calibration, for any of the drugs considered and for this reason the drugs were not assessed using the liquid-liquid extraction procedure. A possible explanation for the irregular trends is that the preliminary data was misleading. For all the drugs considered the preliminary emission measurements appeared to be fairly close together for the differing ratios of drugs to methylene blue, i.e., reproducibility was poor.

![Mean Fluorescence Intensity](image)

**Fig. 4.2:** Calibration of DL propranolol, sodium cromoglycate and warfarin with methylene blue (pH 2.5, citrate) using manual liquid-liquid extraction.

*NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.2. Other parameters: Concentration of methylene blue (pH 2.5, citrate) = 2μM; excitation and emission slits = 5nm; λ<sub>e</sub>=655nm; λ<sub>em</sub>=664nm.*
4.2.2 Methylene blue using an aqueous phase of pH 4 (acetate buffer).

The fluorescence (excitation and emission) spectra (Fig. 4.3) is shown for the organic phase after methylene blue (acetate buffer, pH 4), was shaken with chloroform. Preliminary fluorescence emission data to determine the extractability of methylene blue (pH 4, acetate buffer) and named drugs into chloroform is shown in Fig. 4.4 using manual liquid-liquid extraction.

Fig. 4.3: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when methylene blue (pH 4, acetate) was shaken with chloroform using manual liquid-liquid extraction.

It can be seen from Fig. 4.4, the changes in the measured signals for amitryptilline, digitoxin, nedocromil, sulphadiazine and warfarin make them suitable for further investigation, i.e., calibration with methylene blue (pH 4, acetate buffer) using manual liquid-liquid extraction (Fig. 4.5).
Fig. 4.4: Fluorescence emission data obtained to determine the extractability of methylene blue and named drugs (pH 4, acetate buffer) using manual liquid-liquid extraction.

NB: Concentration of methylene blue = 2μM; Slits of spectrofluorimeter 5nm.
Fig. 4.5: Calibration of amitryptilline, digitoxin nedocromil, sulphadiazine and warfarin with methylene blue pH 4 (acetate) using manual liquid-liquid extraction.

NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.5. Other parameters: Concentration of methylene blue (pH 4, acetate) = 2μM; excitation and emission slits = 5nm; λ<sub>e</sub> = 655nm; λ<sub>em</sub> = 664nm.
The graphical representation (Fig. 4.5) for the calibration does not show a trend with respect to any of the drugs considered using manual liquid-liquid extraction. The irregularity in the trends may be due to the high background fluorescence due to the free fluorophore also being extracted into chloroform, interfering with the signal of the fluorophore/drug complex. As a result of the poor trends flow extraction studies were not pursued.

4.2.3 Methylene blue using an aqueous phase of pH 6.9 (mes buffer).

Fig. 4.6 shows the fluorescence (excitation and emission) spectrum for the organic phase after shaking methylene blue (pH 6.9, mes buffer) with chloroform. Preliminary data to determine the extractability of methylene blue (pH 6.9, mes buffer) and named drugs into chloroform is shown in Fig. 4.7.

![Fluorescence spectrum](image)

**Fig. 4.6:** Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase after methylene blue (pH 6.9, mes buffer) was shaken with chloroform using manual liquid-liquid extraction.

Differences in the signals for amitryptilline, DL propranolol, nedocromil, sulphameth oxazole and warfarin with methylene blue (pH 6.9, mes buffer) were seen (Fig. 4.7) compared to the blank suggesting that it may be possible to calibrate the system with respect to the drugs. These were considered for calibration using manual liquid-liquid extraction (Fig. 4.8).
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<td>Sulphameth oxazole</td>
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<tr>
<td>Warfarin</td>
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</table>

**Fig. 4.7:** Fluorescence emission data to determine the extractability of methylene blue (pH 6.9, mes buffer) and named drugs with chloroform using manual liquid-liquid extraction.

*NB: Concentration of methylene blue = 2μM; Slits of spectrofluorimeter 5nm.*
Fig. 4.8: Calibration of amitryptilline, nedocromil, DL propranolol, sulphameth oxazole and warfarin with methylene blue (pH 6.9, mes buffer) using manual liquid-liquid extraction.

*NB:* All the measurements were made in triplicate, only the mean values are shown in Fig. 4.8. Other parameters: Concentration of methylene blue (pH 6.9, mes) = 2μM; excitation and emission slits = 5nm; λ<sub>ex</sub> = 655nm; λ<sub>em</sub> = 664nm.
From the graphical representation given (Fig. 4.8), it can be seen that only warfarin gave a trend that was worth pursuing using on-line liquid-liquid extraction. The calibration with respect to mean peak area is shown in Fig. 4.9.

![Graph showing calibration for warfarin/methylene blue (pH 6.9, mes buffer), with on-line liquid-liquid extraction.](image)

**Fig. 4.9:** Calibration for warfarin/methylene blue (pH 6.9, mes buffer), with on-line liquid-liquid extraction.

*NB: The measurements were made in triplicate, only the mean values are shown in Fig. 4.9. Other parameters: Concentration of methylene blue (pH 6.9, mes) = 20μM; excitation and emission slits = 5nm; λ<sub>e</sub>=655nm; λ<sub>e</sub>=664nm.*

Fig. 4.9 illustrates that methylene blue (pH 6.9, mes buffer) could be used to detect warfarin using on-line liquid-liquid extraction. Studies in Chapter Five will show whether or not the analysis is suitable when warfarin is present in human serum.
4.2.4 Methylene blue using an aqueous phase of pH 10 (borate buffer).

The fluorescence (excitation and emission) spectrum of the organic phase after methylene blue (pH 10, borate buffer) was shaken with chloroform is given in Fig. 4.10. Preliminary fluorescence emission data to determine the extractability of methylene blue (pH 10, borate buffer) and named drugs into chloroform is given in Fig. 4.11.

![Fluorescence spectrum](image)

**Fig. 4.10:** Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase for methylene blue (pH 10, borate) shaken with chloroform using manual liquid-liquid extraction.

From Fig. 4.11, barbitone, digitoxin, flufenamic acid, phenylbutazone and warfarin show changes in the fluorescence emission signal warranting further investigation when the drug:methylene blue ratio signals were compared to those of the blank. Fig. 4.12 represents the calibration data graphically.
Fig. 4.11: Fluorescence emission data to determine the extractability of methylene blue (pH 10, borate buffer) and named drugs into chloroform using manual liquid-liquid extraction.

*NB:* Concentration of methylene blue = 2μM; Slits of spectrofluorimeter 5nm.
Fig. 4.12: Calibration of barbitone, digitoxin, flufenamic acid, phenylbutazone and warfarin with methylene blue (pH 10, borate buffer) extracted into chloroform using manual liquid-liquid extraction.

NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.12. Other parameters: Concentration of methylene blue (pH 10, borate) = 2μM; excitation and emission slits = 5nm; λ<sub>e</sub> = 655nm, λ<sub>em</sub> = 664nm.
Barbitone and digitoxin did not exhibit a trend (Fig. 4.12) worth pursuing with on-line liquid-liquid extraction. Flufenamic acid, phenylbutazone and warfarin, however, exhibited trends which were investigated using on-line liquid-liquid extraction. Fig. 4.13 shows the calibration data for flufenamic acid, phenylbutazone and warfarin using the flow extraction manifold, the linearity indicating that these drugs are suitable for on-line liquid-liquid extraction.

![Graph showing calibration data for flufenamic acid, phenylbutazone, and warfarin.](image)

**Fig. 4.13:** Calibration for flufenamic acid, phenylbutazone and warfarin, with methylene blue (pH 10, borate buffer) using on-line liquid-liquid extraction.

*N.B.: The measurements were made in triplicate, only the mean values are shown in Fig. 4.13. Other parameters: Concentration of methylene blue (pH 10, borate) = 20µM; excitation and emission slits = 5nm; λ_ex = 655nm; λ_em = 664nm.*
Fig. 4.13 illustrates that methylene blue (pH 10, borate buffer) can be used to detect flufenamic acid, phenylbutazone and warfarin using the on-line liquid-liquid extraction manifold suggested. Studies in Chapter Five will show whether or not the analysis is suitable when these drugs are present in human serum.

4.3 Rhodamine 800.
Rhodamine 800 (structure shown) has been employed in lasers\textsuperscript{152,153}. It has the advantages of exhibiting a stable fluorescence in aqueous media and is not subject to photodecomposition in the presence of light. As explained in Chapter 3, section 3.3.1, preliminary work involved shaking 5ml of the fluorophore and drug in an aqueous solution with 5ml of chloroform. The absorbance and fluorescence (excitation and emission) spectra of the decanted organic layer after shaking with chloroform is measured and the data recorded is used to determine whether or not to pursue the study of the particular drug/fluorophore combination.

![Rhodamine 800](image)

Rhodamine 800 (M. Wt.=496.5)

4.3.1 Rhodamine 800 using an aqueous phase of pH 2.5 (citrate buffer).
Fig. 4.14 shows the fluorescence (excitation and emission) spectrum of the organic phase when Rhodamine 800 (pH 2.5, citrate buffer) was shaken with chloroform. The fluorescence emission data obtained to determine the extractability of Rhodamine 800 (pH 2.5, citrate buffer) and named drugs with chloroform is shown in Fig: 4.15.
Fig. 4.14: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when Rhodamine 800 (pH 2.5, citrate) was shaken with chloroform using manual liquid-liquid extraction.

Considering the trends in Fig. 4.15, amitryptilline, barbitone, nedocromil and warfarin give data which suggested further investigation would be suitable. Fig 4.16 shows the mean fluorescence emission intensities obtained when these drugs were used for calibration using manual liquid-liquid extraction with Rhodamine 800 (pH 2.5, citrate buffer) as the fluorophore.
Fig. 4.15: Fluorescence emission data to determine the extractability of Rhodamine 800 (pH 2.5, citrate buffer) and named drugs into chloroform using manual liquid-liquid extraction.

*NB: Concentration of Rhodamine 800 = 2μM; Slits of spectrofluorimeter 5nm.*
Fig. 4.16: Calibration of amitryptiline, barbitone, nedocromil and warfarin with Rhodamine 800 (pH 2.5, citrate buffer) using manual liquid-liquid extraction.

NB: The measurements were made in triplicate, only the mean values are shown in Fig 4.16. Other parameters: Concentration of Rhodamine 800 (pH 2.5, citrate) = 2 μM; excitation and emission slits = 5nm; λₑ = 624nm; λₑm = 698nm.
There are no apparent trends (Fig. 4.16) seen with respect to the fluorescence intensities when considering amitryptilline, barbitone, nedocromil or warfarin with Rhodamine 800 (pH 2.5, citrate buffer), as a result flowing studies were not pursued.

4.3.2 Rhodamine using an aqueous phase of pH 4 (acetate buffer).

The fluorescence (emission and excitation) spectra for the organic phase when Rhodamine 800 (pH 4, acetate) was shaken with chloroform is shown in Fig. 4.17. The data to determine the extractability of Rhodamine 800 (pH 4, acetate) and named drugs using manual liquid-liquid extraction is given in Fig. 4.18.

![Image](image-url)

**Fig. 4.17:** Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when Rhodamine 800 (pH 4, acetate) was shaken with chloroform using manual liquid-liquid extraction.
Fig. 4.18: Fluorescence emission data to determine the extractability of Rhodamine 800 (pH 4, acetate buffer) and named drugs extracted with chloroform using manual liquid-liquid extraction.

NB: Concentration of Rhodamine 800 = 2μM; Slits of spectrophotometer 5nm.
From the data in Fig. 4.18, flufenamic acid, nedocromil and warfarin show changes in the signals which warrant further investigation. Fig. 4.19 gives the calibration of the drugs with Rhodamine 800 (pH 4, acetate buffer).

![Graph showing calibration of drugs](image)

Fig. 4.19: Calibration for flufenamic acid, nedocromil and warfarin with Rhodamine 800 (pH 4, acetate buffer) using manual liquid-liquid extraction.

*NB: The measurements were made in triplicate, only the mean values are shown in Fig. 4.19. Other parameters: Concentration of Rhodamine 800 (pH 4, acetate) = 2 mM; excitation and emission slits = 5 nm; λ<sub>ex</sub> = 624 nm; λ<sub>em</sub> = 698 nm.*

Nedocromil and warfarin did not give suitable calibrations with manual liquid-liquid extraction, however flufenamic acid gave a trend that was pursued using on-line liquid-liquid extraction. The calibration for flufenamic acid using on-line liquid-liquid extraction is shown in Fig. 4.20.
Mean Peak Area

[ Flufenamic acid ] / mM

Fig. 4.20: Calibration of flufenamic acid with Rhodamine 800 (pH 4, acetate buffer) using on-line liquid-liquid extraction.

NB: The measurements were made in triplicate, only the mean values are shown in Fig. 4.20. Other parameters: Concentration of Rhodamine 800 (pH 4, acetate) = 20μM; excitation and emission slits = 5nm; \( \lambda_{ex} = 680\text{nm}; \lambda_{em} = 698\text{nm} \).

As can be seen from Fig. 4.20 a linear calibration for flufenamic acid is obtained indicating that it could be detected using on-line liquid-liquid extraction. Further studies will be carried out in Chapter Five to establish whether or not this manifold can be used when flufenamic acid is present in human serum.

4.3.3 Rhodamine 800 using an aqueous phase of pH 6.9 (mes buffer).

Fig. 4.21 shows the fluorescence (excitation and emission) spectrum for the organic phase after shaking Rhodamine 800 (pH 6.9, mes buffer) with chloroform. The fluorescence data to determine the extractability of Rhodamine 800 (pH 6.9, mes buffer) into chloroform using manual extraction is given in Fig. 4.22.
Fig. 4.21: Fluorescence (excitation (A) and emission (B)) spectrum for Rhodamine 800 (pH 6.9, mes) shaken with chloroform using manual liquid-liquid extraction.

Fig. 4.22 indicates that amitryptilline, flufenamic acid, nedocromil, phenylbutazone, DL propranolol, sodium cromoglycate, sulphameth oxazole and warfarin should be pursued with respect to a calibration using manual liquid-liquid extraction as there is a significant change in signal for the fluorophore/drug combination compared to the blank. Fig. 4.23 gives the data obtained for the calibration of these drugs with Rhodamine 800 (pH 6.9, mes buffer) using manual liquid-liquid extraction.
Fig. 4.22: Fluorescence emission data to determine the extractability of Rhodamine 800 (pH 6.9, mes buffer) and named drugs extracted with chloroform using manual liquid-liquid extraction.

*NB: Concentration of Rhodamine 800 = 2μM; Slits of spectrofluorimeter 5nm.*
Fig. 4.23: Calibration of amitryptiline, flufenamic acid, nedocromil, phenylbutazone, DL propranolol, sodium cromoglycate, sulphameth oxazole and warfarin with Rhodamine 800 (pH 6.9, mes buffer) using manual liquid-liquid extraction.

NB: The measurements were made in triplicate, only the mean values are shown in Fig. 4.23. Other parameters: Concentration of Rhodamine 800 (pH 6.9, mes) = 2μM; excitation and emission slits = 5nm; λex = 624nm; λem = 698nm.
Only flufenamic acid and warfarin gave a linear calibration when considering manual liquid-liquid extraction and these were considered for on-line extraction. The data obtained for the measurements using the flow extraction manifold is represented graphically in Fig. 4.24.

![Graph showing calibration of flufenamic acid and warfarin](image_url)

**Fig. 4.24:** Calibration of flufenamic acid and warfarin with Rhodamine 800 (pH 6.9, mes buffer) using on-line liquid-liquid extraction.

*NB: The measurements were made in triplicate, only the mean values are shown in Fig. 4.24. Other parameters: Concentration of Rhodamine 800 (pH 6.9, mes) = 20μM; excitation and emission slits = 5nm; λ<sub>x</sub> = 680nm; λ<sub>e</sub> = 698nm.*

A suitable calibration was exhibited (Fig. 4.24) indicating that the manifold could be used for the detection of warfarin and flufenamic acid, the effects of the presence of these drugs in human serum is investigated in Chapter Five.
4.3.4 Rhodamine 800 using an aqueous phase of pH 10 (borate buffer).

The fluorescence (excitation and emission) spectrum for the organic phase when Rhodamine 800 (pH 10, borate buffer) was shaken with chloroform using manual extraction is given in Fig. 4.25. Preliminary data to determine the extractability of Rhodamine 800 (pH 10, borate buffer) and named drugs into chloroform is shown in Fig. 4.26.

![Fluorescence spectrum](image)

Fig. 4.25: Fluorescence (excitation (A) and emission (B)) spectrum for Rhodamine 800 (pH 10, borate) shaken with chloroform using manual liquid-liquid extraction.

*NB: Concentration of Rhodamine 800 = 2μM; Slits of spectrofluorimeter 5nm.*

From the fluorescence emission data given in Fig. 4.26, amitryptilline, phenylbutazone, DL propranolol and sulphamethoxazole were considered for calibration using manual extraction (Fig. 4.27).
Fig. 4.26: Fluorescence emission data to determine the extractability of Rhodamine 800 (pH 10, borate buffer) and named drugs extracted with chloroform using manual liquid-liquid extraction.

*NB: Concentration of Rhodamine 800 = $2\mu M$; Slits of spectrofluorimeter $5\text{nm}$. 
Fig. 4.27: Calibration data for amitryptilline, phenylbutazone, DL propranolol and sulphameth oxazole with Rhodamine 800 (pH 10, borate buffer) using manual liquid-liquid extraction.

NB: The measurements were made in triplicate, only the mean values are shown in Fig. 4.27. Other parameters: Concentration of Rhodamine 800 (pH 6.9, mes) = 20μM; excitation and emission slits = 5nm; λ<sub>ex</sub> = 680nm; λ<sub>em</sub> = 690nm.
It can be seen from Fig. 4.27 that there is no trend with any of the drugs considered and therefore these were not considered for flow extraction studies. Possible explanations of the lack of trend are explained in section 4.2.1.

4.4 3,3' Diethyloxocarbocyanine iodide (DODC).

3,3' Diethyloxocarbocyanine iodide (DODC) (structure shown) has been used as a laser fluorophore\textsuperscript{152}.

\[
\text{DODC (M. Wt. = 486.35)}
\]

The fluorescence (excitation and emission) spectra is shown in Fig. 4.28 for the organic extract, after shaking a solution of DODC (pH 2.5, citrate buffer) with chloroform. Although a significant fluorescence signal was observed for the extract, the fluorescence signal of the aqueous phase (pH 2.5, citrate) was seen to decay rapidly, i.e., the fluorescence emission signal ($\lambda_{em} = 610\text{nm}; \text{slits 2.5nm}$) was seen to decrease from 324.3 to 6.75 arbitrary fluorescence units over a period of 30 mins. This would, even if all the aqueous solutions were prepared immediately prior to extraction pose difficulties when considering the flow extraction studies.
Although a decrease in the emission signal was also seen with an aqueous phase of pH 4 (acetate buffer) and pH 6.9 (mes buffer) the decrease in the signals of 18.6% and 3.6% respectively, over 30 mins, were not considered significant and these were considered for further studies. With an aqueous phase of pH 10 (borate buffer) no decrease in the signal was seen over the same period.

4.4.1 DODC using an aqueous phase of pH 4 (acetate buffer).

Fig. 4.29 shows the fluorescence (excitation and emission) spectra for the organic phase when DODC (pH 4, acetate buffer) was shaken with chloroform. Fig. 4.30 shows the fluorescence emission data to determine the extractability of DODC (pH 4, acetate buffer) and named drugs when extracted with chloroform using manual liquid-liquid extraction.
Fig. 4.30: Fluorescence emission data obtained to determine the extractability of DODC (pH 4, acetate buffer) and named drugs using manual extraction.

NB: Concentration of DODC = 0.2μM; Slits of spectrofluorimeter 2.5nm.
As a result of the data shown in Fig. 4.30, amitryptilline, barbitone, diazepam, flufenamic acid, nedocromil, phenylbutazone, sodium cromoglycate, sulphadiazine and sulphameth oxazole with DODC (pH 4, acetate) were considered for calibration using manual liquid-liquid extraction (Fig. 4.31). As no trend was observed for any of the drugs considered they were not investigated using on-line liquid-liquid extraction.

![Mean Fluorescence Intensity](image)

**Fig. 4.31:** Calibration of named drugs with DODC (pH 4, acetate) using manual liquid-liquid extraction.

*NB: All the measurements were made in triplicate, only the mean values are show in Fig. 4.31. Other parameters: Concentration of DODC = 0.2μM (pH 4, acetate); λ<sub>α</sub> = 590nm; λ<sub>m</sub> = 610nm; Slits 2.5nm.*
4.4.2 DODC using an aqueous phase of pH 6.9 (mes buffer).

Fig. 4.32 shows the fluorescence (excitation and emission) spectra for the organic phase when DODC (pH 6.9, mes buffer) was shaken with chloroform.

![Fluorescence spectra](image)

Fig. 4.32: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when DODC (pH 6.9, mes buffer) was shaken with chloroform.

Fig. 4.33 shows the preliminary data to determine the extractability of DODC and named drugs, (pH 6.9, mes buffer) after extraction into chloroform using manual liquid-liquid extraction. Only phenylbutazone and sodium cromoglycate gave encouraging information and a calibration was attempted using manual liquid-liquid extraction. The data measured for the calibration is shown in Fig 4.34. As a trend was not seen flowing studies were not pursued.
Fig. 4.33: Fluorescence emission data obtained to determine the extractability of DODC and named drugs (pH 6.9, mes buffer) using manual extraction.

*NB: Concentration of DODC = 0.2µM; Slits of spectrofluorimeter 2.5nm.*
Fig. 4.34: Calibration of phenylbutazone and sodium cromoglycate, with DODC (pH 6.9, mes buffer) using manual liquid-liquid extraction.

NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.34. Other parameters: Concentration of DODC (pH 6.9, mes) = 0.2μM; Slits = 2.5nm; λex = 590nm; λem = 610nm.

4.4.3 DODC using an aqueous phase of pH 10 (borate buffer).

Fig. 4.35 shows the fluorescence (excitation and emission) spectra for the organic phase, when DODC (pH 10, borate buffer) was shaken with chloroform. Fig. 4.36 shows the fluorescence emission data obtained for the determination of the extractability of DODC and named drugs (pH 10, borate buffer) using manual liquid-liquid extraction.

Fig. 4.35: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when DODC (pH 10, borate buffer) was shaken with chloroform.
Fig. 4.36: Fluorescence emission data obtained to determine the extractability of DODC and named drugs (pH 10, borate buffer) using manual extraction.

NB: Concentration of DODC = 0.2μM; Slits = 2.5nm.
Considering the data given in Fig. 4.36 amitryptilline, diazepam, phenylbutazone, DL propranolol and warfarin were considered for calibration using manual liquid-liquid extraction. The calibration, using manual liquid-liquid extraction is given in Fig. 4.37.

![Mean Fluorescence Intensity vs. [Drug]/μM](image)

**Fig. 4.37:** Calibration of amitryptilline, diazepam, DL propranolol, phenylbutazone and warfarin with DODC (pH 10, borate buffer) using manual liquid-liquid extraction.

*NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.37. Other parameters: Concentration of DODC (pH 10, borate) = 0.2μM; Slits 2.5nm; λ<sub>e</sub> = 590nm; λ<sub>m</sub> = 610nm.*
It can be seen from Fig. 4.37 that amitryptilline, diazepam and DL propranolol do not give trends worth pursuing, phenylbutazone and warfarin however warrant further investigation and a calibration was attempted using flow extraction studies with DODC (pH 10, borate buffer). The calibration for phenylbutazone and warfarin using the flow extraction manifold is given in Fig. 4.38.

![Diagram](image_url)

**Fig. 4.38:** Calibration of phenylbutazone and warfarin, with DODC (pH 10, borate buffer) with on-line liquid-liquid extraction.

*NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.38. Other parameters: Concentration of DODC (pH 10, borate) = 0.2µM; Slits 2.5nm; λ<sub>ex</sub> = 590nm; λ<sub>em</sub> = 610nm.*

A suitable calibration was exhibited (Fig. 4.38) indicating that the manifold could be used for the detection of phenylbutazone and warfarin, the effects of the presence of these drugs in human serum is investigated in Chapter Five.
4.5 1,1',3,3,3',3'-Hexamethylindotricarbocyanine Perchlorate (HIDTCP).

HIDTCP (structure shown), has been used with manual liquid-liquid extraction previously\(^{141}\).

![HIDTCP structure](image)

HIDTCP (M. Wt. = 590.05)

4.5.1 HIDTCP using an aqueous phase of pH 2.5 (citrate buffer).

Fig. 4.39 shows the fluorescence (excitation and emission) spectra for the organic phase when HIDTCP (pH 2.5, citrate buffer), was shaken with chloroform. Fig. 4.40 shows the fluorescence emission data for the determination of extractability for HIDTCP (pH 2.5, citrate buffer), and named drugs extracted into chloroform.

![Fluorescence spectra](image)

Fig. 4.39: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when HIDTCP (pH 2.5, citrate buffer) was shaken with chloroform.
Fig. 4.40: Fluorescence emission data obtained to determine the extractability of HIDTCP (pH 2.5, citrate buffer) and named drugs using manual liquid-liquid extraction.

*NB: Concentration of HIDTCP = 2μM; Slits = 5nm.*
From the data given in Fig. 4.40, barbitone, diazepam, digitoxin, phenylbutazone, sulphamethoxazole with HIDTCP (pH 2.5, citrate buffer) were considered for calibration (Fig. 4.41). It must be noted that upon increasing the concentration of the drugs a decrease in the signal was seen when compared to the blank measurement, this was not considered to be disadvantageous as long as a calibration could be made with respect to a change in signal. This decrease in signal was not unexpected as Roberson et al., who had previously used HIDTCP for the analysis of surfactants expressed results as a change in signal as opposed to measurements recorded.

From the trends displayed in Fig. 4.41, barbitone, diazepam, digitoxin, phenylbutazone and sulphamethoxazole were considered for assay using the flow extraction manifold. Unfortunately, on-line liquid-liquid extraction gave no observable signals for any of the drugs considered. This could have been due to either the fluorophore/drug complex being too large to pass through the membrane or the concentration of the complex being lower than the limit of detection for the flow extraction manifold i.e., it was not possible to detect the complex using the flow cell with an illuminated volume of 18µl. The latter explanation could have been tested by increasing the slit widths of the LS50, thereby increasing the illuminated volume, however this would have increased the amount of stray light present and possibly an artificial signal may have resulted. The porosity of the membrane could have been increased to allow the complex to pass, this was not viable in this case as the porosity the teflon tape is not known, and membranes with predetermined porosity are expensive and if used would increase the cost of the manifold significantly.
Mean Fluorescence Intensity

Fig. 4.41: Calibration of barbitone, diazepam, digitoxin, phenylbutazone and sulphamethoxazole, with HIDTCP (pH 2.5, citrate buffer) using manual liquid-liquid extraction.

NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.41. Other parameters: Concentration of HIDTCP (pH 2.5, citrate) = 2μM; Slits = 5nm; λ<sub>ex</sub> = 750nm; λ<sub>em</sub> = 782nm.
4.5.2 HIDTCP using an aqueous phase of pH 4 (acetate buffer).

Fig. 4.42 shows the fluorescence (excitation and emission) spectra obtained for the organic phase after HIDTCP (pH 4, acetate buffer) was shaken with chloroform.

![Fluorescence spectra](image)

Fig. 4.42: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when HIDTCP (pH 4, acetate buffer) was shaken with chloroform.

Fig. 4.43 gives the preliminary data observed for the determination of extractability of named drugs and HIDTCP (pH 4, acetate) extracted into chloroform. Only nedocromil was considered for calibration using manual liquid-liquid extraction with HIDTCP (pH 4, acetate buffer), the calibration is given in Fig. 4.44. A trend appeared to be present at a lower concentration range for nedocromil and on-line studies were carried out. However, no measurable signals were observed. A possible explanation is given in section 4.5.1.
Fig. 4.43: Fluorescence emission data to determine the extractability of HIDTCP (pH 4, acetate buffer) and named drugs using manual liquid-liquid extraction.

*NB: Concentration of HIDTCP = 2µM; Slits = 5nm.*
Fig. 4.44: Calibration of nedocromil with HIDTCP (pH 4, acetate buffer), using manual liquid-liquid extraction.

NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.44. Other parameters: Concentration of HIDTCP (pH 4, acetate) = 2μM; Slits = 5nm; λ_ex = 750nm; λ_em = 782nm.

4.5.3 HIDTCP using an aqueous phase of pH 6.9 (mes buffer).

Fig. 4.45 shows the fluorescence (excitation and emission) spectra of the organic phase produced after shaking HIDTCP (pH 6.9, mes buffer) with chloroform. Fig. 4.46 shows the fluorescence emission data to determine the extractability of HIDTCP and named drugs (pH 6.9, mes buffer) into chloroform using manual liquid-liquid extraction.

Fig 4.45: Fluorescence (excitation (A) and emission (B)) spectrum for the organic phase when HIDTCP (pH 6.9, mes buffer) was shaken with chloroform.
Fig. 4.46: Fluorescence emission data to determine the extractability of HIDTCP (pH 6.9, mes buffer) and named drugs using manual liquid-liquid extraction.

NB: Concentration of HIDTCP = 2μM; Slits = 5nm.
Examination of the data given in Fig. 4.46 led to only phenylbutazone being considered for calibration using manual liquid-liquid extraction (Fig. 4.47).

![Graph showing calibration of phenylbutazone with HIDTCP (pH 6.9, mes buffer), using manual liquid-liquid extraction.](image)

**Fig. 4.47:** Calibration of phenylbutazone with HIDTCP (pH 6.9, mes buffer), using manual liquid-liquid extraction.

*NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.47. Concentration of HIDTCP (pH 6.9, mes) = 2μM; excitation and emission Slits = 5nm; λ_ex = 750nm; λ_em = 782nm.*

Phenylbutazone was thus considered for on-line liquid-liquid extraction, the mean peak areas obtained for fluorescence emission are shown in Fig. 4.48. A suitable calibration was exhibited (Fig. 4.48) indicating that the manifold could be used for the detection of phenylbutazone, the effects of the presence of phenylbutazone in human serum is investigated in Chapter Five. The fact that a calibration has been possible using on-line liquid-liquid extraction, nullifies the prediction about the fluorophore/drug complex being too large to pass through the membrane given in section 4.5.1.
Fig. 4.48: Calibration of phenylbutazone with HIDTCP (pH 6.9, mes buffer), using on-line liquid-liquid extraction.

NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.48. Other parameters: Concentration of HIDTCP (pH 6.9, mes) = 20μM; excitation and emission slits = 5nm; λ_{ex} = 750nm; λ_{em} = 782nm.

4.5.4 HIDTCP using an aqueous phase of pH 10 (borate buffer).

The fluorescence (excitation and emission) spectra is shown in Fig. 4.49 for the organic phase for HIDTCP (pH 10, borate buffer) shaken with chloroform. Fig. 4.50 gives the fluorescence emission data to determine the extractability of named drugs with HIDTCP (pH 10, borate buffer) into chloroform.

Fig 4.49: Fluorescence (excitation (A) and emission(B)) spectrum for the organic phase when HIDTCP (pH 10, borate buffer) was shaken with chloroform.
Fig. 4.50: Fluorescence emission data to determine the extractability of HIDTCP (pH 10, borate buffer) and named drugs using manual liquid-liquid extraction.

NB: Concentration of HIDTCP = 2μM; Slits = 5nm.
From the data given in Fig. 4.50 phenylbutazone and sodium cromoglycate gave encouraging information, a calibration with HIDTCP (pH 10, borate buffer) using manual liquid-liquid extraction was carried out (Fig. 4.51).

![Calibration of phenylbutazone and sodium cromoglycate with HIDTCP (pH 10, borate buffer), using manual liquid-liquid extraction.]

**Fig. 4.51:** Calibration of phenylbutazone and sodium cromoglycate with HIDTCP (pH 10, borate buffer), using manual liquid-liquid extraction.

*NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 4.51. Other parameters: Concentration of HIDTCP (pH 10, borate) = 2 μM; excitation and emission slits = 5 nm; λ<sub>ex</sub> = 750 nm; λ<sub>em</sub> = 782 nm.*

Although sodium cromoglycate (Fig. 4.51) did not give a suitable trend, phenylbutazone did. A suitable calibration using on-line liquid-liquid extraction was obtained for phenylbutazone (Fig. 4.52), the effects of the presence of phenylbutazone in human serum is investigated in Chapter Five.
Fig. 4.52: Calibration of phenylbutazone with HIDTCP (pH 10, borate buffer), using on-line liquid-liquid extraction.

*NB:* All the measurements were made in triplicate, only the mean values are shown in Fig. 4.52. Other parameters: Concentration of HIDTCP (pH 10, borate) = 20 μM; excitation and emission slits = 5 nm; λ<sub>exc</sub> = 750 nm; λ<sub>em</sub> = 782 nm.

4.6 Conclusion.

It is evident from the work presented in Chapter Four, that a suitable liquid-liquid extraction-flow injection manifold has been suggested to determine certain drugs after their extraction into an organic phase from the aqueous phase. However, there were also systems which although give suitable calibrations with manual liquid-liquid extraction, did not do so upon injection onto the flow extraction manifold. This could have simply due to the sensitivity of the technique, i.e., it was not possible to detect the complex using the flow cell with an illuminated volume of 18 μl. The latter explanation could have been tested by increasing the slit widths of the LS50, thereby increasing the illuminated volume. However this would have increased the amount of stray light present and possibly an artificial signal may have resulted. The porosity of the membrane could have been increased to allow the complex to pass, this was not viable in this case as the porosity of the teflon tape is not known, and membranes with pre-determined porosity are expensive and if used would increase the cost of the manifold significantly.
CHAPTER FIVE

INVESTIGATION OF LIQUID-LIQUID EXTRACTION PROCESSES
5.1 Introduction.
Having established an on-line liquid-liquid extraction method which can be used to determine various drugs with fluorimetric detection in the long wavelength region, this chapter will describe what the extraction involves, i.e., whether or not the extraction is pH or ion dependent, whether or not ion pairing takes place and also if the on-line liquid-liquid extraction procedure could be used with serum samples.

5.2 Effect of ions and pH on the fluorescence emission signal.
To determine whether or not the aqueous phase had any effect on the fluorescence emission signal, two studies were carried out. The first was to determine whether the ions present in the buffer influenced the fluorescence emission signal. This was carried out by injecting drug/fluorophore combinations known to give a signal onto the manifold, and the mean peak area compared to signals obtained upon replacing the buffer containing different ions, of the same pH. The data recorded is shown in Table 5.1.

Table 5.1: Mean peak area data to determine the effect of ions in the aqueous phase during extraction using on-line liquid-liquid extraction.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Mean fluorescence emission peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIDTCP/Phenylbutazone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>2.5</td>
<td>239.4</td>
</tr>
<tr>
<td>Chloride</td>
<td>2.5</td>
<td>2079.5</td>
</tr>
<tr>
<td>Rhodamine 800/Flufenamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>4</td>
<td>289.2</td>
</tr>
<tr>
<td>Phthalate</td>
<td>4</td>
<td>185.4</td>
</tr>
<tr>
<td>Rhodamine 800/Warfarin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mes</td>
<td>6.9</td>
<td>67.7</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>6.9</td>
<td>18.55</td>
</tr>
</tbody>
</table>

cont/...
cont. Table 5.1

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Mean fluorescence emission peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borate</td>
<td>10</td>
<td>1798.3</td>
</tr>
<tr>
<td>Carbonate</td>
<td>10</td>
<td>1926.7</td>
</tr>
</tbody>
</table>

NB: pH 2.5 [HIDTCP] = 20µM; [Phenylbutazone] = 1mM; Slits ex=em=5nm; λex=750nm; λem=782nm. pH 4 [Rhodamine 800] = 2µM; [Flufenamic acid] = 1mM; Slits ex=5nm; em=10nm; λex=680nm; λem=696nm. pH 6.9 [Rhodamine 800] = 2µM; [Warfarin] = 1mM; Slits ex=5nm; em=10nm; λex=680nm; λem=696nm. pH 10 [HIDTCP] = 20µM; [Phenylbutazone] = 600µM; Slits ex=em=5nm; λex=750nm; λem=782nm.

From Table 5.1 it is obvious that the type of ions present in the aqueous phase do affect the fluorescence signal. It appears that for the majority of the aqueous phases considered the preferred one was chosen. An aqueous phase of pH 2.5, chloride buffer unfortunately gave considerably better signals than the pH 2.5, citrate buffer. Chloride buffer was not considered with a view to the extraction measurements as it is well documented that the presence of chloride ions quench the fluorescence signal\(^\text{153}\). Secondly, the effect of pH on the fluorescence emission signal was examined (Table 5.2), the pH was adjusted by one or two units keeping the pH within the working range of the buffer.

Table 5.2: Mean peak area data to determine the effect of pH of the aqueous phase during extraction using on-line liquid-liquid extraction.

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean fluorescence emission peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No measurable signal</td>
</tr>
<tr>
<td>2.5</td>
<td>239.4</td>
</tr>
<tr>
<td>3</td>
<td>192.1</td>
</tr>
</tbody>
</table>

cont/...
From the data shown in Tables 5.1 and 5.2 it is evident that in the analyses considered pH control and choice of buffer are significant. For the system considered the most appropriate choices of buffer were made in the majority of cases. It is clear that the extractions are pH as well as ion dependent.

5.3 Ion pairing or fluorophore extraction?

To establish whether ion pairing were being produced or alternatively more or less fluorophore was extracted in the presence of various drugs was investigated. This was determined using manual liquid-liquid extraction. The fluorophore was initially present in the organic phase as opposed to the aqueous phase as considered for the experimentation in Chapter Four. The aqueous phase contained the drug in buffer known to give a change in signal. Measurements
of the organic phase were made before and after shaking with the aqueous phase and are recorded in Table 5.3.

Table 5.3: Data representing the signals obtained before and after shaking when the fluorophore is present initially in the organic phase and the drug in the aqueous phase, using manual liquid-liquid extraction.

<table>
<thead>
<tr>
<th></th>
<th>Absorption</th>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \lambda_{\text{abs}} )</td>
<td>( \lambda_{\text{ex}} )</td>
<td>Intensity</td>
</tr>
<tr>
<td>Methylene Blue (2µM)/Warfarin (4µM) pH 10, borate buffer.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shaking</td>
<td>652</td>
<td>654</td>
<td>139.5</td>
</tr>
<tr>
<td>No shaking</td>
<td>652</td>
<td>654</td>
<td>97.5</td>
</tr>
<tr>
<td>HIDTCP (2µM)/Phenylbutazone (4µM) pH 10, borate buffer.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shaking</td>
<td>754</td>
<td>748</td>
<td>200.5</td>
</tr>
<tr>
<td>No shaking</td>
<td>758</td>
<td>748</td>
<td>339.1</td>
</tr>
<tr>
<td>DODC (0.2µM)/Warfarin (0.4µM) pH 10, borate buffer.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shaking</td>
<td>589</td>
<td>591</td>
<td>103.4</td>
</tr>
<tr>
<td>No shaking</td>
<td>589</td>
<td>591</td>
<td>113.4</td>
</tr>
<tr>
<td>Rhodamine 800 (2µM)/Warfarin (4µM) pH 6.9, mcs buffer.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shaking</td>
<td>684</td>
<td>682</td>
<td>932</td>
</tr>
<tr>
<td>No shaking</td>
<td>684</td>
<td>683</td>
<td>705.9</td>
</tr>
</tbody>
</table>

NB: Slit Widths - Methylene Blue: \( \text{ex} = \text{em} = 5\text{nm} \). HIDTCP: \( \text{ex} = \text{em} = 5\text{nm} \). DODC: \( \text{ex} = \text{em} = 2.5\text{nm} \). Rhodamine 800: \( \text{ex} = \text{em} = 5\text{nm} \). \( \text{ex} = \text{excitation and em} = \text{emission} \).

In order to determine which process was taking place an inspection of the absorption intensities was made. If ion pairing was occurring the absorption intensities before and after shaking would not alter significantly. This would be accompanied by a change in fluorescence emission intensity. The absorption intensities did not alter drastically with DODC and methylene blue i.e., ion pairing was taking place. The change in the absorption signals for HIDTCP and Rhodamine 800 was quite marked, i.e., more or less fluorophore was being extracted in the presence of the drug. In the case of HIDTCP where, for calibration, the data was seen to decrease upon increasing the concentration.
of the drug, can be explained by the fact that the drug/fluorophore complex favours the aqueous phase whereas the fluorophore alone favours the organic phase, i.e., the drug/fluorophore complex is hydrophilic.

5.4 Serum samples.

Having confirmed an analysis to carry out on-line liquid-liquid extraction, it was important to determine whether or not the manifold could be used to determine drugs contained in human serum. No clean up procedure of the serum was undertaken before injection onto the manifold, in order to establish the extent of interference, if any, from any of the constituents of the serum that may affect the fluorescence signal.

DODC, with an aqueous phase of pH 10 (borate buffer), was used to carry out the preliminary work. Initial measurements (Table 5.4) were made extracting DODC/phenylbutazone/serum and also DODC/serum using the flow extraction manifold.

Table 5.4: Measurement of the mean peak area of the fluorescence emission signal in the presence and absence of phenylbutazone to determine the extent of any interference from human serum.

<table>
<thead>
<tr>
<th>DODC: serum</th>
<th>Mean peak area of fluorescence emission signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Presence of drug</td>
</tr>
</tbody>
</table>
| Phenylbutazone | 1912 | -
| 1:500        | 1707 | 922 |
| 1:200        | 2712 | 1518 |
| 1:100        | 2014 | 1439 |
| 1:50         | 1619 | 1906 |
| 1:20         | -2250| 2195 |

NB: [DODC] = 2μM; [Phenylbutazone] = 600μM; Aqueous phase pH 10, borate buffer, λex = 590nm; λem = 610nm; Slits ex = em = 5nm.
The presence of serum obviously affects the fluorescence emission. The signal increases with increasing concentration of human serum. The large negative mean peak area obtained for a 1:20 dilution in the presence of phenylbutazone suggests that fouling of the membrane by the constituents of the serum has possibly occurred, i.e., the pores of the membrane are blocked, with the DODC/serum/phenylbutazone complex no longer being able to pass through the membrane. Another explanation could be that the serum, phenylbutazone and DODC form a complex at the higher concentration of serum that is extracted into the organic phase but the fluorescence is somehow masked thus giving a quenched signal. Individual drug/fluorophore combinations were also examined for the effect of serum, however, only two ratios of fluorophore/serum were considered (Table 5.5). In addition, the fluorophore was also injected in the absence of the drug, and the signals observed in the presence of the serum measured, these are also shown in Table 5.5.

The data given in Table 5.5 indicates that in the presence of the drug/fluorophore, the fluorescence signal is affected by the presence of the serum, however the signal is not necessarily affected in the absence of the drug, i.e., methylene blue, pH 10 (borate buffer), methylene blue, pH 6.9 (mes buffer) and Rhodamine 800 pH 4 (acetate). This indicates that the serum does not actually cause any interference with respect to the fluorophore, suggesting that the serum and drug may form a complex which in turn interferes with the fluorescence signal.

The interference observed (Table 5.5) may take the form of the serum/drug complex not complexing with the fluorophore thereby less drug is present for the fluorophore to complex with i.e., quenching of the signal. A further explanation is the drug/serum complex prefers to remain in the aqueous phase or once the serum/drug/fluorophore complex is formed, the fluorophore becomes "trapped" within the complex thereby quenching the signal.
Table 5.5: Observed mean peak area upon addition of human serum to drug/fluorophore complexes using on-line liquid-liquid extraction.

<table>
<thead>
<tr>
<th>Fluorophore/Drug</th>
<th>pH of carrier stream</th>
<th>In the presence of Drugs</th>
<th>In the absence of Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No serum</td>
<td>1:200</td>
<td>1:20</td>
</tr>
<tr>
<td>Methylene Blue/ Phenylbutazone</td>
<td>10</td>
<td>426.1</td>
<td>396.5</td>
</tr>
<tr>
<td>Methylene Blue/ Flufenamic acid</td>
<td>10</td>
<td>383.6</td>
<td>421.2</td>
</tr>
<tr>
<td>Methylene Blue/ Warfarin</td>
<td>10</td>
<td>486.5</td>
<td>479.5</td>
</tr>
<tr>
<td>Methylene Blue/ Warfarin</td>
<td>6.9</td>
<td>134.9</td>
<td>115.1</td>
</tr>
<tr>
<td>Rhodamine 800/ Flufenamic acid</td>
<td>4</td>
<td>715.5</td>
<td>196.3</td>
</tr>
<tr>
<td>Rhodamine 800/ Flufenamic acid</td>
<td>6.9</td>
<td>166.7</td>
<td>514.7</td>
</tr>
<tr>
<td>Rhodamine 800/ Warfarin</td>
<td>6.9</td>
<td>284.5</td>
<td>622.4</td>
</tr>
<tr>
<td>DODC/ Phenylbutazone</td>
<td>10</td>
<td>581.9</td>
<td>896.9</td>
</tr>
<tr>
<td>DODC/ Warfarin</td>
<td>10</td>
<td>1156.7</td>
<td>1324.7</td>
</tr>
<tr>
<td>HIDTCP/ Phenylbutazone</td>
<td>6.9</td>
<td>-1135.6</td>
<td>-1263.5</td>
</tr>
<tr>
<td>HIDTCP/ Phenylbutazone</td>
<td>10</td>
<td>-2746.1</td>
<td>-3139.3</td>
</tr>
</tbody>
</table>

NB: [Methylene Blue] = [HIDTCP] = 20μM; [Rhodamine 800] = [DODC] = 2μM; [Warfarin] Methylene Blue = [Flufenamic acid] = 1mM; [Phenylbutazone] = [Warfarin] DODC, Rhodamine 800 = 600μM; Slits ex = em = 5nm except for Rhodamine 800 slits ex = 5nm; em = 10nm. Methylene Blue λ_ex = 655nm; λ_em = 664nm; Rhodamine 800 λ_ex = 680nm; λ_em = 696nm; DODC λ_ex = 590nm; λ_em = 610nm; HIDTCP λ_ex = 750nm; λ_em = 782nm. 1:200 and 1:20 indicate the ratio of the fluorophore to human serum.
The corresponding signals for HIDTCP (pH 10, borate buffer), DODC (pH 10, borate buffer) and Rhodamine 800 (pH 6.9, mes buffer) are shown to be influenced by the presence of the serum, suggesting that the fluorophore and serum form a complex. The interference in the case of Rhodamine 800 appears to be pH dependent.

5.4.1 Calibration in the presence of human serum.

To establish whether a calibration for warfarin using DODC (pH 10, borate buffer) as the fluorophore was carried out, the calibration obtained for the peak area of the emission signal in the absence of serum, 1:200 (fluorophore:serum); and 1:20 (fluorophore:serum) is shown in Fig 5.1.

Fig. 5.1 shows that although it would not be possible to determine warfarin when serum is present in a 1:20 (fluorophore: serum molar ratio). There do not appear to be any difficulties when the serum is present in a molar ratio of 1:200 (fluorophore:serum). This trend would, in theory, be predicted for the other fluorophore/drug combinations studied.

5.5 Conclusion.

It has been discovered that the processes taking place in the manifold were pH and ion dependent (sections 5.2 and 5.3). There does not appear to be a "hard and fast rule" as to which mechanism the fluorophore/drug complexes give a change in signal. DODC and Methylene Blue were shown to undergo ion pair extraction, whereas with Rhodamine 800 and HIDTCP the concentration of the fluorophore extracted altered with a change in the drug concentration. Finally, the advantages of the long wavelength region were clearly exhibited upon determining drugs in low levels of human serum. A linear calibration was obtained for a 1 fluorophore:200 human serum molar ratio, within the therapeutic range of the drug.
Fig. 5.1: Calibration of warfarin and DODC with and without serum.

NB: All the measurements were made in triplicate, only the mean values are shown in Fig. 5.1. [DODC] = 2μM; λ\text{ex} = 590 nm; λ\text{em} = 610 nm; Slits ex-em = 5 nm. 1:200 and 1:20 indicate the ratio of the fluorophore to human serum.
CHAPTER SIX

CONCLUSIONS
6.1 Conclusions.

A number of fluorophores (methylene blue, Rhodamine 800, DODC, and HIDTCP) were studied to investigate the possibility of their uses as agents to complex with the drugs considered (Appendix), which could then be extracted from the aqueous phase into the organic phase using on-line and manual liquid-liquid extraction.

Fluorescence measurements in the long wavelength region (550-1000nm) are a recent development in photoluminescence spectroscopy and offer many advantages compared with conventional measurements recorded in the ultraviolet and visible spectral regions. These include, reduced background fluorescence in the presence of biological materials; reduced scattering; decreased photodecomposition and the availability of inexpensive, solid state optical components which operate in the long wavelength spectral region.

Both manual and on-line liquid-liquid extractions have been shown to be effective for the determination of flufenamic acid, phenylbutazone and warfarin within their therapeutic ranges, i.e., 7.8mM-11.7mM; 0.32-0.65mM and 0.16-2.7mM respectively. Suitable linearity (Table 6.1) was obtained for all the drug/fluorophore combinations considered. The on-line procedure was also found suitable for the determination of drugs when present in low level concentrations of human serum, highlighting one of the major advantages of long wavelength fluorophores of reduced background fluorescence. Studies have shown that the fluorescence signals are strongly dependent upon the type of buffer (Chapter Five, section 5.1), as well as the ions present. It was found that the correct choice of buffer was made in the majority of cases.
Table 6.1: Correlation coefficients for different drug/fluorophore combinations using on-line liquid-liquid extraction.

<table>
<thead>
<tr>
<th>Drug/Fluorophore</th>
<th>pH of carrier stream</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue/Phenylbutazone</td>
<td>10</td>
<td>0.992</td>
</tr>
<tr>
<td>Methylene Blue/Flufenamic acid</td>
<td>10</td>
<td>0.994</td>
</tr>
<tr>
<td>Methylene Blue/Warfarin</td>
<td>10</td>
<td>0.990</td>
</tr>
<tr>
<td>Methylene Blue/ Warfarin</td>
<td>6.9</td>
<td>0.997</td>
</tr>
<tr>
<td>Rhodamine 800/Flufenamic acid</td>
<td>4</td>
<td>0.994</td>
</tr>
<tr>
<td>Rhodamine 800/Flufenamic acid</td>
<td>6.9</td>
<td>0.999</td>
</tr>
<tr>
<td>Rhodamine 800/Warfarin</td>
<td>6.9</td>
<td>0.987</td>
</tr>
<tr>
<td>DODC/Phenylbutazone</td>
<td>10</td>
<td>0.956</td>
</tr>
<tr>
<td>DODC/Warfarin</td>
<td>10</td>
<td>0.945</td>
</tr>
<tr>
<td>HIDTCP/Phenylbutazone</td>
<td>6.9</td>
<td>0.994</td>
</tr>
<tr>
<td>HIDTCP/Phenylbutazone</td>
<td>10</td>
<td>0.952</td>
</tr>
</tbody>
</table>

On-line liquid-liquid extraction has been shown to be advantageous with respect to low sample volume consumption; rapid rates of analysis and minimization of operator contact with the extracting solvent when compared with manual extraction. The design of the phase separator was such that it gave an efficiency of separation of 96%. The efficiency being determined as the ratio of the signals for identical solutions without the phase separator in the manifold and with the phase separator placed in-line. The mechanism of extraction was found to be ion pairing for DODC and methylene blue whereas for Rhodamine 800 and HIDTCP a greater or lesser proportion of the dye was extracted into the organic phase depending upon the concentration of the drug present (Chapter 5, section 5.2).

The use of chloroform was not considered "user friendly". Its carcinogenic properties are disadvantageous even though on-line extraction permits
minimum operator contact with the extracting solvent. Although many solvents exist which may be utilised for extraction studies, the disadvantages of many of these is their density. The on-line procedure considered, dictated that the extracting solvent be of greater density than the aqueous phase. This fact reduces the commonly used list of extracting solvents significantly. Dichloromethane and carbon tetrachloride were shown (Chapter 3, section 3.3.1) to present difficulties. Two solvents of greater density than the aqueous phase, that were considered for extraction were ethylacetoacetate (density = 1.025g/ml) and dimethylmaleonate (density = 1.154g/ml). On-line liquid-liquid extraction using dimethylmaleonate was shown to give much reduced sensitivity compared to chloroform (density = 1.47g/ml). This may have been due to the solvent being reactive, thereby possibly causing some non-fluorescent reactions, as opposed to chloroform which is fairly inert in comparison. Ethylacetoacetate was found to be unsuitable for on-line extraction, as the membrane disintegrated upon attempting to pass the solvent through it. Manual liquid-liquid extraction was found not to give a calibration using either of the solvents.

This project has shown that on-line liquid-liquid extraction is possible in the long wavelength region using the manifold described. The technique, in addition to the advantages of on-line analysis, has the benefits of sensitivity and specificity of fluorescence as well as all the advantages of measurement in the long wavelength region. Work has involved the use of various fluorophores with different drugs. Further studies could be carried out to improve the efficiency of the phase separator i.e., the use of commercially available membranes, e.g., Millipore, Goretex etc., this would unfortunately be accompanied by an increase in the total cost of the analysis. Investigation into an alternative extracting solvent should also be considered. Simple manual liquid-liquid extraction procedures could be carried out on-line, and possibly automated. Complex manual procedures could also be considered with modified versions of the manifold. As new long wavelength fluorophores are developed, they could also be used to develop the technique further.
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<th>Journal and Volume</th>
<th>Year</th>
<th>Page</th>
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Amitryptilline
*Cyclic antidepressant*

Barbitone
*Sedative*

Diazepam
*Minor Tranquillizer*

Digitoxin
*Cardiotonic*

Flufenamic acid
*Anti-inflammatory*
Nedocromil
*Anti-asthmatic*

Phenylbutazone
*Anti-inflammatory*

Propranolol
*Non selective β-receptor antagonist*

Sodium cromoglycate
*Anti-asthmatic*

Sulphadiazine
*Anti-bacterial*

Sulphamethoxazole
*Anti-bacterial*
Warfarin
*Anti-coagulant*