DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-BASED TOXICOLOGICAL SCREENING PROCEDURE FOR NEUTRAL AND ACIDIC DRUGS IN THE URINE OF POISONED PATIENTS

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BY

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Abbreviations

a.u. - Absorbance units

a.u.f.s. - Absorbance units full scale

DAD - Photo diode array detector

GC - Gas chromatography

GC-FTIR - Gas chromatography / Fourier transform infrared spectrophotometry

GC-MS - Gas chromatography / Mass spectrometry

GLC - Gas liquid chromatography

HPLC - High performance liquid chromatography

IS - Internal standard

ISA - Internal standard solution A

ISB - Internal standard solution B

LC - Liquid chromatography

mAU - Milli absorbance units

MPCA - Mobile phase component A

MPCB - Mobile phase component B

NPD - Nitrogen phosphorus selective detector

ODS - Octadecylsilane

RI - Retention index

RRT - Relative retention times

SPE - Solid phase extraction

TEAP - Tetraethyl ammonium phosphate

TLC - Thin layer chromatography

UV - Ultraviolet

VIS - Visible

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Summary

The necessity of developing broadly based in-house toxicology screening procedures and their associated databases in any department purporting to offer a toxicology service, cannot be overestimated. The development, in the past twenty years, of a number of different immunoassay techniques with which a large number of target compounds can be detected very selectively, all too often lulls the physician treating a drug overdose case into a false sense of security once a specific compound has been identified. This is so because, once a drug has been identified which appears to account for the patient's condition, the search for other possible contributors is more often than not, abandoned.

The aim of this study was to develop a screening procedure for neutral and acidic drugs in the urine of poisoned patients to complement the already existing gas chromatographic screening procedure for basic drugs. Sixty different substances were identified as a nucleus with which to launch the project. These substances were selected on the basis of their availability to the public and on data of overdose cases treated in this laboratory during the past twenty three years.

Information on a large number of published screening procedures for these type of compounds and the various techniques involved in their isolation, detection and identification, was collected initially. These techniques were subsequently optimised with the aim to develop a combined high performance liquid chromatography / diode array detector / UV spectral database based screening procedure which is simple and can be performed rapidly at reasonable costs. The liquid-liquid extraction procedure for acidic and neutral drugs, with diethyl ether as organic solvent and a clean-up washing step with lead acetate solution, gave good recoveries and yielded chromatograms with little interference by endogenous compounds.

The general applicability of this screening procedure in a series of suspected drug overdoses, has lead to the isolation and identification of a number of drugs added as

standards to the spectral database as well as some unexpected compounds which were subsequently added to the database. Metabolites of several drugs were also identified in the urine of patients in some of the case reports presented. The data on these compounds were likewise added and are still being added to the spectral library whenever new data become available during the treatment of overdose cases. The metabolite patterns of drugs obtained in this way are invaluable additions to the screening procedure as they contribute significantly to making the identification of isolated drugs unambiguous.

The simplicity and speed of the developed screening procedure, makes it ideal for toxicological screening since the whole procedure can be performed in under an hour. Because a simple liquid-liquid extraction procedure is used, the costs are less than normally encountered with solid phase extraction methods. Moreover, it is certainly much cheaper and faster than a series of immunoassay-based target analyses which would be needed to cover even a small fraction of the compounds which can be isolated and identified by this broadly based procedure.

The results obtained in this study demonstrated that selective testing could have grave consequences for the patient. Commercially available quick tests for toxicology screening can be misleading because clinicians can get a false impression of the abilities of laboratories rendering such services. This was illustrated in a number of case studies by using the screening procedure developed during this study. A good example is the case in which theophylline was found during an HPLC screening. On the basis of the patient's history the clinicians only requested target analyses for anticonvulsants of which two were actually found in the patient's plasma and of which one was at a concentration which could account for the patient's clinical condition. The high plasma level of theophylline assayed subsequent to theophylline being detected in the stomach content, could have been fatal.

It can be concluded that this project yielded a comprehensive and versatile screening method and database for the identification of acidic and neutral drugs in urine. The data obtained during several of the case studies contributed information which was useful in the treatment of the patients involved.

Opsomming

Die noodsaaklikheid vir die ontwikkeling van 'n omvattende binnenshuise toksikologiese siftingsprosedure en geassosieerde databasis in enige departement wat voorgee om 'n toksikologiediens te lewer, kan nie oorbeklemtoon word nie. Die ontwikkeling oor die afgelope twintig jaar van 'n verskeidenheid immunologiese bepalingmetodes, waarmee 'n groot aantal teikenverbindings baie selektief opgespoor kan word, sus die klinikus in die behandeling van 'n oordoseringsgeval in 'n valse gevoel van versekerdheid, wanneer 'n spesifieke middel geïdentifiseer is. Wanneer 'n geneesmiddel wat moontlik die pasiënt se toestand kan verklaar, eers geïdentifiseer is, word die soektog vir ander moontlike bydraende faktore dikwels nie uitgeskakel nie.

Die doel van die studie was om ter aanvulling van bestaande gaschromatografiese siftingsprosedures vir basiese geneesmiddels, 'n siftingsprosedure vir suur- en neutrale geneesmiddels in die urien van vergiftigde pasiënte te ontwikkel. Sestig verskillende substanse is geïdentifiseer as 'n kern, waarmee die projek begin is. Die substanse is gekies op die basis van hulle beskikbaarheid aan die publiek en data van oordoseringsgevalle wat gedurende die afgelope drie-en-twintig jaar in hierdie laboratorium versamel is.

Inligting oor 'n groot getal gepubliseerde siftingsprosedures vir hierdie tipe verbindings asook verskeie tegnieke betrokke in die isolasie, opsporing en identifikasie, is aanvanklik versamel. Hierdie tegnieke is voorts geöptimiseer met die doel om 'n gekombineerde hoë-verrigtings-vloeistof-chromatografie / dioderangskikkingsdetektor / UV-spektra-databasis-gebaseerde siftingsprosedure, wat eenvoudig is en wat vinnig uitgevoer kan word teen 'n lae koste, te ontwikkel. Die vloeistof-vloeistof ekstraksieprosedure vir suur en neutrale geneesmiddels, met diëtieleter as organiese oplosmiddel en 'n skoonmaakprosedure met loodasetaat, het goeie opbrengste gelewer en chromatogramme met min inmenging deur endogene verbindings is verkry.

Die algemene toepasbaarheid van die onderhawige siftingsprosedure, in 'n reeks ondersoeke van die urien asook ander liggaamsvloeistowwe van pasiente wat opgeneem is vir die behandeling van vermeende geneesmiddeloordoserings, het aanleiding gegee tot die isolasie en identifikasie van verskeie geneesmiddels wat oorspronklik as standaarde tot die databasis gevoeg is, terwyl 'n aantal onverwagte nuwe verbindings bykomend tot die databasis gevoeg is. Metaboliete van verskeie geneesmiddels is ook in die urien van pasiënte in sekere van die genoemde gevallestudies gevind. Die data van hierdie verbindings is tot die spektrumbiblioteek toegevoeg en sal deurlopend bygevoeg word wanneer nuwe data beskikbaar word gedurende die behandeling van oordoseringsgevalle. Die metabolietpatrone van geneesmiddels wat op die wyse bekom word, is waardevolle toevoegings tot die siftingsprosedure, omdat dit bydra tot die onomwonde identifikasie van die geïsoleerde geneesmiddels.

Die eenvoudigheid en spoed van die metode wat ontwikkel is maak dit ideaal vir 'n toksikologiese siftingsprosedure, omdat die hele prosedure binne 'n uur uitgevoer kan word. Die metode behels 'n eenvoudige vloeistof-vloeistof ekstraksieprosedure, wat tot gevolg het dat kostes laer is as wat normaalweg met soliede fase ekstraksiemetodes ondervind word. Die metode is beslis baie goedkoper en vinniger as die reeks van imunologies-gebasseerde teikenbepalings, wat normaalweg nodig sou wees om selfs 'n klein fraksie van die verbindings, wat geïsoleer en geïdentifiseer kan word, te dek.

Die resultate wat in die studie verkry is, het dan ook getoon dat selektiewe toetsing ernstige nagevolge vir 'n pasiënt kan inhou. Kommersiële beskikbare kitstoetse vir toksikologiesiftingsprosedures kan misleidend wees, omdat klinici 'n vals indruk van die vermoëns van die laboratorium wat die dienste verskaf kan kry. Laasgenoemde is in 'n verskeidenheid van gevallestudies, aan die hand van die siftingsprosedure wat in hierdie studie ontwikkel is, geïllustreer. 'n Goeie voorbeeld is die geval waarin teofillien tydens 'n hoë-verrigtings-vloeistof-chromatografie sifting gevind is. Gebaseer op die pasiënt se geskiedenis, het die klinici slegs teikenanalises vir anti-konvulsante versoek, waarvan

twee werklik in die pasiënt se plasma gevind is. Een van die verbindings het 'n plasmakonsentrasie, waaraan die pasiënt se kliniese toestand toegeskryf kon word, weerspieël. Die hoë plasmakonsentrasie van teofillien wat egter later bepaal is, nadat dit deur middel van die siftingsprosedure in die maaginhoud opgespoor is, kon fataal gewees het.

Die gevolgtrekking kan gemaak word dat hierdie studie 'n omvattende en veelsydige siftingsprosedure en databasis vir die identifikasie van suur- en neutrale geneesmiddels in urien opgelewer het. Die data verkry gedurende verskeie van die gevallestudies, het bygedra tot betekenisvolle inligting wat nuttig was in die behandeling van die betrokke pasiënte.

CHAPTER 1

Introduction

Among the many drugs which have entered the market, the number of analgesic and non-steroidal anti-inflammatory drugs made available to the public during the past two decades has increased tremendously. Most of these drugs are available as over the counter preparations and are thus quite freely available to the public without prescription. Coupled with the fact that analgesic drugs are known to be misused to a large extent by the general public, the frequency with which these drugs are identified during overdose cases has increased; be it as the main component of the overdose or merely as a complicating accompaniment with the overdose of another drug substance. Additionally and in general, over the counter drugs are carelessly handled in many households, are often stored within reach of children and are thus implicated in a large percentage of the unintentional drug overdose cases seen by casualty departments.

When a doctor is confronted with an overdose, an attempt must be made to determine the kind of drug or substance involved, as soon as possible. The development of a more specific target screening procedure for these drugs as an adjunct to the existing general thin layer chromatographic screening and the basic drug gas chromatographic screening procedures, was therefore considered to be desirable.

In the light of the proceeding introductory remarks, the more specific purpose of this study was to develop a suitable screening procedure for the detection and identification of analgesic and non-steroidal anti-inflammatory drugs, available on the market in the Republic of South Africa, in the urine of poisoned patients.

Rapid and accurate analysis of the body fluid samples of the poisoned patient requires intensive investigation techniques, especially in the case of multiple drug ingestion. Various analytical techniques are available for the analysis of such samples. In general the determination of drugs in biological matrix is divided into two stages namely:

1. sample preparation and isolation followed by

2. the actual identification of the drug.

The isolation or recovery of drugs from the biological matrix can be achieved by various extraction techniques, for example:

1. Liquid-liquid extraction, which has an acceptable recovery, is relatively cheap and can be rapid depending on the circumstances and the sample matrix.

2. Solid phase extraction which, despite being a powerful tool, is limited because of the costs involved as well as the nature of the biological samples used; for instance in forensic work, whole blood tends to clog the solid phase extraction column. Because of their potential for selectivity, most solid phase extraction methods have been focused on individual drugs or groups of related drugs and only recently serious attempts have been made to extend the use of solid phase extractions to class-independent screening procedures in toxicological work.

Except for immunoassays, most analytical toxicology procedures require isolation of the drugs from either an aqueous or biological matrix (usually plasma or urine) prior to the actual measurement. A very important aspect of any isolation procedure is the extent of the recovery of the analyte from the matrix since this will often determine whether a component will be detected; especially when one deals with some of the modern potent drugs. Most published analytical procedures usually contain a report of recovery and in this respect all the extraction procedures mentioned earlier have been reported on in the publications cited. Since no single extraction procedure can be singled out as being the preferred procedure for all drugs the choice of isolation procedure used is often dictated by parameters such as availability, convenience, speed etc. In the case of emergency toxicology, for instance, where one often deals with overdoses of drugs, speed of isolation is more often than not, more important than good recovery.

The determination or identification of the substance, once it has been isolated, can be done by a variety of techniques in which chromatography plays the most important central role.

Since a very high percentage of drugs contain nitrogen, gas chromatography (GC) offers high sensitivity and selectivity in drug analysis especially when a nitrogen phosphorus selective detector (NPD) is used. However, acidic drugs, and also the more polar drugs, do not chromatograph well on GC, and derivatization to form a less polar and more volatile compound is often necessary. The advent of HPLC offers another option in the analysis of these drugs.

In a relatively short time HPLC has become a widely used, fully established and efficient analytical technique. The rapid development is attributable to the progress in column and instrumental technologies. With well-designed data handling systems HPLC has been greatly expanded and is particularly suitable for drugs which are polar, for example analgesic and the non-steroidal anti-inflammatory drugs. The development of diode array detectors and the introduction in 1982, by Hewlett-Packard, of the world's first commercially available diode array detector, has been one of the most important advances in HPLC in the last 10 years because it tremendously increased the scope for identifying substances eluting from the column. The improvement of HPLC pumps has also led to better chromatography and the achievement of more reproducible flowrates meant that more reliable retention times could be achieved.

However, reproducibility of retention time data between laboratories is recognised as being a problem in HPLC methodology because of small changes resulting from local conditions e.g. water, reagent quality, columns etc. For these reasons it is recommended that individual laboratories prepare their own database of retention times and UV spectra.

Because of the nature of the drugs to be identified (i.e. mostly acidic and neutral compounds), it was decided to opt for screening of the drugs in urine using a single liquid-liquid extraction while incorporating a simple clean-up step followed by gradient HPLC with diode array UV detection. It was postulated that this HPLC-based toxicological screening prosedure with accompanying extraction procedure, as developed in this study, would prove to be a reliable procedure to identify neutral and acidic drugs in the urine of poisoned patients.

CHAPTER 2

Literature Review

Traditionally toxicological extracts were screened for non-volatile organic compounds by a tedious process of systematic colour and crystal tests (Steward and Stolman, 1961).

Ultraviolet absorption and its change with pH have been used extensively as a systematic approach to the presumptive identification of compounds to which they are applicable. Serious limitations are imposed on this procedure because, for a mixture of components, a composite spectrum for all absorbing compounds is obtained.

Paper chromatography has also been used successfully to screen tissue extracts (Steward and Stolman, 1961). In the latter case the resulting fractions were traditionally located and characterised by an array of chromogenic reagents and could be eluted, depending on previous treatment, for further examination. Many different chromatographic solvent systems were, however, required for adequate separation within the acidic, basic, and neutral fractions that resulted from toxicological extractions.

During the 1960's, thin layer chromatography (TLC) replaced paper chromatography as an extremely valuable adjunct for detection of drugs in screening procedures, are presently in use and will probably still continue to be used for a long time to come. TLC is rapid and ideally suited for drug identification. It is a technique which can be easily introduced for only a small capital outlay and its application to the analysis of urine specimens is particularly commendable since in some cases characteristic spot patterns are produced by the drugs and their metabolites. The literature abounds with drug screening procedures based on this technique with the most extensive compilation of data useful to the analytical toxicologist being the second, revised and enlarged edition of "Thin-layer Chromatographic Rf Values of Toxicological Relevant Substances on Standardised Systems". The latter is a special issue of the TIAFT Bulletin designated as Report XVII of the DFG Commission for Clinical-Toxicological Analysis and prepared

under the auspices of the Committee for Systematic Toxicological Analysis of TIAFT by de Zeeuw et al. (1992 a).

Soon after the advent of gas chromatography (GC) in the 1950's, its remarkable utility was amply illustrated by its many applications to complex analyses as it was immediately applied by many toxicologists and analysts in other biomedical fields (Ettre, 1959). Initially the applicability of gas chromatography to routine analysis was complicated by a variety of column substrate materials and different operating conditions reported. This problem was addressed by Parker et al. (1963) who described a gas chromatographic method for the screening of toxicological extraction residues for alkaloids, barbiturates, sympathomimetic amines, and tranquillisers. Two Carbowax 20 M (alkaline and non alkaline) columns were compared to determine their usefulness in the screening of toxicological extracts and retention time data were given for 41 alkaloids chromatographed on an SE-30 column at five temperatures. In the same year Kazyak and Knoblock (1963) presented gas chromatographic data on compounds of general toxicological interest with a discussion on the problems of application of this technique to routine analysis using SE-30 and QF-1 columns. GC became a cornerstone in many laboratories because it offered remarkable operational efficiency in the screening process and identification of negative analyses.

Numerous publications supplying gas chromatographic reference data pertinent to toxicology followed but few, if any, were directly linked to a simple and systematic scheme of analysis. Finkle et al. (1971) presented a simple GC system for the identification of a wide range of materials encountered in forensic toxicology which formed the foundation of an efficient screening system. They used four columns and three liquid phases, complemented by a direct solvent extraction scheme designed to detect common poisons, drugs and human metabolites to a sensitivity of 2 µg/ml in blood, urine, and tissue specimens. Relative retention data for almost 600 different substances were tabulated in two tables, one being an alphabetical name index and the

other a relative retention time index. Thus, one table provided reference GC information for any of the substances prior to the analysis while the other allowed tentative identification of unknown GC peaks.

Automated drug analysers equipped with automatic liquid samplers and integrators on-line with a data processor were soon developed by Solon (1972). The automatic liquid sampler and integrator allowed one to obtain extremely precise retention time and areas, thus allowing the operator to do a rapid qualitative and/or quantitative screen for compounds which were not completely resolved. The system was capable of screening urine extracts at the rate of one sample every six minutes. The data processor with the appropriate software added the capability of combining the results from several analysers into a single report.

Owing to the work of Moffat et al. (1974) and Moffat (1975), it was soon generally accepted that dimethylsilicone phases such as SE-30 and OV-1 provide optimum discriminating power and that measurement of retention indices Kovats (1958) "Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone" is the technique of choice for substance characterisation as well as for the compilation of gas chromatographic data in a data bank and the exchange of those data between different laboratories. Schepers et al. (1982), extended the work done with retention indices of drugs on packed columns to capillary columns and also chose to work with temperature programmed runs to diminish analysis times. Presently the most extensive compilation of GC data useful to the analytical toxicologist is the third, revised and enlarged edition of "Gas Chromatographic Retention Indices of Toxicological Relevant Substances on Packed or Capillary Columns with Dimethylsilicone Stationary Phases". The latter is a special issue of the TIAFT Bulletin and designated as Report XVIII of the DFG Commission for Clinical-Toxicological Analysis prepared under the auspices of the Committee for Systematic Toxicological Analysis of TIAFT by de Zeeuw et al. (1992).

The development of more selective detectors, such as the nitrogen/phosphorus selective detector, has gone a long way in addressing the problems of differentiation between nitrogen-containing exogenous compounds and many endogenous non-nitrogen-containing compounds such as lipids, fatty acids and metabolites of food substances excreted in urine. To increase the reliability of GC analysis even more, emphasis was soon placed on supplemental qualitative techniques such as infrared spectrophotometry (GC-FTIR) and mass spectrometry (GC-MS). Initially the additional expertise and expense incurred by these techniques limited their usefulness in situations calling for large workloads, but these limitations have now been overcome to a large extent by many commercially available instruments. The second, revised and enlarged edition of "Mass Spectral and GC data of Drugs, Poisons, Pesticides, Pollutants and their Metabolites" by Pfleger et al. (1992) is representative of the best compilation of GC-MS data available to the analytical toxicologist at present.

Prior to 1967, liquid column chromatography was considered as slow and inefficient. However, the modernised technique now commonly known as high performance liquid chromatography (HPLC), has undergone dramatic changes and now rivals GC in speed of analysis and column efficiency. Initially there existed a common misconception that HPLC is less efficient than GC. Giddings (1965) was one of the first to recognise that the theory developed for GC was equally applicable, with minor modifications, to HPLC. The theory indicated that by using high pressures and very small particles, HPLC could rival GC both in speed and resolving power. In the case of GC, long columns can be used to increase the number of theoretical plates. The much lower permeability of HPLC columns, due to the very small particle size and the much higher viscosities of liquids, places a severe limitation on the column length but this is more than compensated for by the greater selectivity of this mode of separation; very difficult separations being achievable even with relatively short volumes. The greater selectivity is achieved through the choice of mobile phase, solvent programming and variations in the separation mechanism; i.e. adsorption, ion exchange and partitioning.

An important benefit of the application of the above mentioned theory was a unification of the practice of liquid chromatography. Modern HPLC equipment is similar for all forms of liquid chromatography and is equally usable for adsorption, partition ion exchange and gel chromatography. The most decisive feature of current HPLC is the use of highly sensitive as well as selective detectors. Initially the refractive index monitor and the ultraviolet (UV) spectrophotometer were the most popular but there are many principles on which detection may be based, and these have been and are still being extensively researched and exploited. Klatt (1979) published the first report of an isolation technique for unresolved peaks based on a rapid scanning multiwavelength detection system interfaced with a microcomputer. At the time the system was very costly and required a high degree of skill. In the five years following this publication, the cost of microcomputers and photodiode arrays were drastically reduced and became readily available. Multiwavelength UV detectors with up to 256 and more element photodiode arrays became commercially available during that time and attracted more chromatographers because they offered great advantages for qualitative analysis. HPLC, using gradient elution and photodiode array detection, was quickly adopted by many toxicologists as an extremely useful drug screening procedure for specific classes of drugs.

Since it was the aim of this project to develop and set up an HPLC screening procedure with diode array detection for neutral and acidic drugs in urine samples from suspected poisoned patients, this literature survey will further concentrate, in more detail, on this aspect of drug screening. Emphasis was placed on the HPLC equipment used as well as the extraction and recovery of screened drugs.

2.1 HPLC Equipment

An excellent review of multielement detectors was published by Talmi (1975). The classical silicon photodiode linear array, manufactured by Reticon, was the first detector marketed successfully. The development of two linear photodiode array spectrometers based on this array and used as detector systems in automated liquid chromatography, was described by Dessy et al. (1976 a). The first system involved a minimum configuration involving a microprocessor and a linear photodiode array acting as a rapid scanning single beam UV/VIS detector. This instrument could monitor up to eight single wavelengths. It plotted the absorption at the selected wavelengths as a function of time on a standard x/y plotter and could take a complete spectrum about every five seconds. The second system was designed around a large minicomputer system and was intended for pattern recognition work focusing on metabolite characterisation, isolation and identification. It was capable of taking 20 spectra/second in a dual-beam mode.

In a similar vein another new mini-computer automated linear photodiode array spectrometer system for high-resolution liquid chromatography was developed by Dessy et al. (1976 b). This third generation multiwavelength array spectrometer was developed as a detector for high-resolution liquid chromatographic characterisation of metabolites. The design included a mini-computer, a matched pair of linear photodiode (256 element) arrays, holographic ruled gratings, fibre optics, flow cells, and a high-intensity xenon light source. The resolution could be independently adjusted in the spectral range of 200 - 800 nm. This system was used for the separation and characterisation of the metabolites of a carcinogen, 4-ethylsulfonyl-1-naphthalenesulfonamide.

A rapid scanning diode array as a multiwavelength detector in liquid chromatography was described by Milano et al. (1976). They demonstrated how first derivative spectra can be gainfully utilised in a unique way to maximise the information obtained from this detector. The detector consisted of a low volume flow cell and real time signal averaging of the spectra, allowed operation at 0.005 a.u.f.s. with noise levels of 0.0005

a.u. peak to peak. The authors demonstrated that photodiode array detectors can allow operation with the sensitivities needed in modern LC. The availability of spectra rather than absorbency at a single wavelength allows better identification and quantitation of the solutes. This additional information can also be used for deconvolution of overlapping peaks by plotting absorbance or first derivative absorbance chromatograms at various wavelengths.

The performance of a silicon target vidicon tube as a multiwavelength detector for liquid chromatography was discussed by Mc Dowell and Pardue (1977). The rapid scanning spectrometer used in this work was based on a silicon diode array vidicon camera tube and a magnetic focus and deflection assembly. This device covered a spectral range from about 200 nm to above 800 nm and permitted scan repetition rates of up to 100 scans per second. The most serious limitation of the vidicon detector system used in this work was the photometric drift which resulted from the single beam operation.

The use of a rapid scanning diode array spectrometer for enhanced sensitivity via first derivative chromatograms was reported by Milano and Grushka (1977). They compared the sensitivity of the absorbency and first derivative measures using data previously collected for mixtures of benzene, benzyl chloride, and anisole. The results indicated that the use of first derivative detection in liquid chromatography did not only allow deconvolution of overlapping peaks, but also reduced the noise due to lamp fluctuations.

Molnar and Horvath (1977) separated over a hundred acidic constituents present in urine. A high-pressure liquid chromatograph with a variable wavelength UV detector at 280 nm or alternatively a fluorescence detector at 260 nm excitation and 340 nm emission wavelengths, was used in gradient elution mode. The constituents were separated within 30 min. by using a 5-μm octadecyl-silica column, with the gradient elution increasing the acetonitrile concentration in dilute aqueous phosphoric acid solution at 70°C. This study demonstrated that many low-molecular-weight constituents

of physiological fluids can be separated faster and more efficiently by microparticular non-polar stationary phases than ion-exchange resins employed conventionally.

Milano et al. (1978) described the characterisation of the photodiode array detector in liquid chromatography. In order to access the viability of the photodiode array detector, its noise characteristics and linear range were studied. It was found that, at worst, the peak noise was ± 0.001 a.u. The detector was found to have a linear range between 2 x 10^3 and 6.5 x 10^3 depending on the wavelength. The linearity of the detector was determined with a mixture of three components namely naphthalene, pyrene and benzopyrene. A mixture of benzene, toluene, p-xylene and mesitylene was used in the study of deconvolution and quantitation of overlapping peaks. The photodiode array detector was found to be linear over a wide range of absorbances with noise characteristics roughly the same as those of other commercial detectors and the vidicon detector. Some problems with the presented system was that stability needed to be improved and that stray light tended to be relatively high with array detectors, as filters and double monochromators cannot be used.

A simultaneous multiwavelength detection system for liquid chromatography which consisted of a rapid-scan spectrometer with a UV silicon vidicon target as radiant energy detector, was used in a study by Klatt (1979). He described algorithms required for real-time data processing capabilities. The use of this detection system to qualitatively assess compositional changes in coal derived oil as a function of process, as well as the limitation of the system and required improvements, were discussed. The results presented indicate that the simultaneous multiwavelength detection system possesses numerous and unique capabilities. However, three principal limitation are apparent. First, the detection limits are poorer than conventional single wavelength monitors. The second limitation concerns the cost of the system and thirdly a skilled operator is required in order to obtain the maximum value from a simultaneous multiwavelength LC detector.

While work on the diode array detectors was progressing, other developments in drug screening techniques also progressed at a rapid rate. A combination of TLC,GLC and HPLC for the rapid detection of drugs and related compounds was compiled by Daldrup et al. (1981). Almost 570 substances were tested by 8 different chromatographic methods. In the case of the HPLC procedure a UV detector at 220 nm was used and an isocratic mobile phase consisting of acetonitrile and phosphate buffer adjusted accurately to pH 2.30 was the preferred mode.

A review of progress in the field of multichannel detection in column and thin-layer chromatography was presented by Fell et al. (1983 a). Some novel applications for a computer-based linear photodiode array UV-visible spectrophotometer for detection in high-performance liquid chromatography, computer-aided methods for simultaneous monitoring of the elution profile at three wavelengths with automatic peak detection and capture of UV spectra for diacetylmorphine and its principal metabolites and degradation products were either discussed or described. The authors found that the optical multichannel detector offers a rapid and versatile approach to detection in HPLC and TLC. By combining retention time data with rapidly scanned spectra captured during elution, drugs, their degradation products and metabolites can be characterised with greater specificity. Peak homogeneity can be examined by use of absorbency ratio and qualitative features can be enhanced.

Novel techniques for peak recognition and deconvolution by computer-aided photodiode array detection in high-performance liquid chromatography were also examined by Fell et al. (1983 b). Digital algorithms for manipulating spectrochromatographic data, obtained by means of photodiode array detectors, were tested for peak purity and for their quantitative performance. Two model systems noscapine and papaverine, and the red pigments R112 and R3, were studied. The absorbency ratio plotted as function of time performed less well in recognising peak overlap than the second derivative of the elution profile. Spectral deconvolution with a "dummy" linear absorbency standard, and a non-

absorbing reference wavelength, permitted the elution profiles of both model systems to be completely resolved. This method revealed the elution profile of an unknown, closely overlapping peak in a sample containing papaverine and noscapine.

On-line screening for urapidil and metabolites in dog urine by high-performance liquid chromatography with a diode array UV detector was achieved by Zech et al. (1983). Because of the similarity of the metabolite spectra to the spectrum of the parent compound, selection was based on absorption ratios. They found that comparison of up-slope, apex and down-slope spectra is a convenient way of testing for peak purity.

In a study by Chan and Chan (1984) an HPLC was fitted with a programmable UV detector, and was used for the analysis of acidic and neutral drugs. The analytical column was a reverse-phase Hypersil ODS 5µm, 200mm x 4.6mm column. A phosphate buffer with a pH of 3.2 was used as a mobile phase and a gradient elution programme with acetonitrile was chosen. The relative retention times remained fairly stable and the initial screening procedure was routinely confirmed by gas chromatography, colorimetry and UV spectrophotometry. The authors were able to screen for all 40 drugs described when their concentrations in blood were in the toxic range.

Hoshino et al. (1984) published results on the use of a photodiode array detector to identify unresolved peaks. He built a multiwavelength UV detector with a 32-element photodiode array and a data processor and used it for the quantitative isolation of poorly resolved chromatographic peaks. He further used a mathematical technique for simultaneous multicomponent analysis of UV-spectra in order to develop computer software for the determination of unknown and hidden components. Three-dimensional chromatographic data was computed by a data processor and then stored on a floppy disk. The results obtained were compared with those obtained by conventional perpendicular dropping and tangential-skimming methods. Recoveries achieved by the new method were 100.7 and 101.1% while those achieved by the conventional methods were 89.9 and 110.1%.

However, the identification of unknown drugs during drug screening procedures does not depend only on the detector. The extraction of the drugs from complex matrices into suitable solvents in which the drug could be further purified and enriched relative to the co-extracted endogenous components, is equally important and so are the many possibilities of utilising the unique selectivity of HPLC.

The analysis of antipyretics using semimicro columns (150 x 1mm I.D.) was studied by Matsushima et al. (1985). The columns used were octadecyl silica and styrene-divinylbenzene porous polymer. The analyte was dissolved in 80% aqueous acetonitrile for the ODS silica column and in methanol for the porous polymer column. Seven antipyretic drugs were chromatographed with a mobile phase of acetonitrile and water (25:75) on the ODS column. With the porous polymer column three mobile phases were used, namely methanol, methanol containing 1% acetic acid and methanol containing 1% aqueous ammonia. The methanol containing 1% aqueous ammonia was most suitable for the separation of a mixture of antipyretics. Xanthine drugs were also separated with the same mobile phase. An HPLC with a UV monitor operating at 254nm was used. The results described indicate that semimicro columns can be used for the separation of antipyretic agents.

A new method of separation and identification of aromatic and nitrogen-containing acids in urine was presented by Ramnaraine and Tuchman (1985). Fifty-seven different acids were separated by reversed-phase HPLC. Detection and identification of the acids by UV spectra using a photodiode array detector (DAD) were demonstrated. They found that this method cannot replace the use of GC in metabolic profiling but that it is a complementary technique for compounds which cannot be determined by GC.

Stevens and Gill (1986) used three eluents to chromatograph analgesics on an ODS silica column. The eluents were prepared by mixing aqueous potassium dihydrogen phosphate with formic acid and isopropanol. The three eluents had isopropanol contents of 1.7%, 15% and 35% respectively. This assay was carried out using an LC UV variable-

wavelength detector operating at 244nm. Quantification was done using peak height ratio measurements with paracetamol as an internal standard. This method proved to be useful for the identification and quantification of analgesics and anti-inflammatory drugs.

Useful sample handling techniques for reversed phase HPLC in emergency toxicology were discussed by Van Damme et al. (1986). Using a multichannel detector, the advantages of dilution as well as liquid and solid extractions were also compared by the authors. In this way they (Van Damme et al., 1986) demonstrated the importance of sample handling in toxicological analyses in which reversed phase HPLC is used. Sodium dihydrogen phosphate buffer with the pH adjusted with acetic acid or phosphoric acid and with different percentages of acetonitrile was used as the mobile phase. Instead of dihydrogen phosphate the authors used sodium acetate in the chromatography of caffeine and theophylline.

A sequential simplex procedure for the optimisation of mobile phase composition in HPLC, combined with multichannel detection, was developed by Wright et al. (1987). An efficient stop criterion for automated simplex procedures, based on continuous comparison of the chromatographic response function attained with that predicted, was proposed by the authors. They concluded that multi-channel detection has a number of features which strengthens its use with sequential simplex optimisation.

HPLC photodiode array UV detection for toxicological drug analysis was applied by Hill and Langner (1987). Retention data for 157 compounds in an acid HPLC system, and 144 compounds in a basic HPLC system were listed. They also listed UV spectral data for 25 compounds of each system. For the analysis of acidic and neutral drugs, a Zorbax C8 column maintained at 31°C was used. Compounds were eluted using a linear solvent program of 0 to 100 % B/A in 30 min. of the following: Solvent A was 0.1 % (v/v) H_3PO_4 and solvent B was $CH_3CN : H_2O$ (9 + 1) containing 0.1 % (v/v) H_3PO_4. The flowrate was 2.0 ml/min. Reference compounds were dissolved in methanol at a concentration of 500 µg/ml and 10 to 40 µl of this solution was analysed. Retention data

were recorded and the UV spectra at the apex of the peaks were normalised and stored in a reference library. The study demonstrated that combining HPLC separation of drugs with UV detection and computer matching of spectra can be an effective analytical tool.

Thirty-seven selected acidic and neutral drugs were analysed by Bogusz and Aderjan (1988) by means of HPLC with a two step gradient elution. Nine different ODS silica columns were evaluated in this study, and the retention index values (RI) were calculated. A series of alkyl-arylketones were used for the calculation of the RI values. The mobile phase, a mixture of phosphate buffer (pH 3.2) and acetonitrile was used to run a gradient. The solvent gradient was 10% acetonitrile at 0 minutes to 70% at 30 minutes. All samples were analysed at 220nm and at ambient temperature.

In the applied gradient elution, the relation between the carbon numbers of the alkyl-arylketones and their capacity factor was linear. The RI values for the examined drugs were therefore calculated using k' values. Standardisation of the retention data by RI alone did not reduce the intercolumn variability of the nine columns tested. The RI values were then corrected with a method described in an earlier publication by Bogusz (1987) . After correction the RI values improved the comparability of the different columns and also increased the accuracy thereof. The authors suggest that when this method is used, the results obtained by HPLC in all laboratories should be comparable.

Traditionally, the optimisation of separation conditions has always been performed by trial -and-error experiments which rely on the experience and intuition of the investigator. However, when an analysis requires a rapid response, this procedure cannot be relied upon. Computer-assisted systems can decrease the analysis response time, especially if the investigator is inexperienced in the field. In this regard a retention prediction approach, based on quantitative-structure retention relationships, has been described by Jinno et al. (1988) and Jinno et al. (1989). Their system determines the candidates of toxic compounds contained in the biological fluid by combining the

retention prediction concept and the UV spectral matching process. The actual components of the system were the following:

1. A retention prediction system which predicted the retention times for particular compound groups such as polycyclic aromatic hydrocarbons, amino acids, small peptides etc.

2. An LC data base which provided the basic information for LC separations.

3. An automated identification system which automatically identified compounds using the retention prediction concept.

4. An automated optimisation system which optimised separation conditions for particular compound groups, such as amino acids, and offered minimum analysis time for maximum resolution for this compound groups and offered minimum analysis time for maximum resolution for the compound group desired.

5. Multi-320:chromatographic data station for the multichannel UV detection system which worked as the data processor to interpret the information obtained by the multichannel UV detector.

6. UV spectral data base system which stored UV spectra published in the literature. The UV data base was used for the spectral matching process to identify the components separated by the liquid chromatograph.

The expert system was constructed on an NEC personal computer PC 9801-VX2 16 bit on a MS-DOS operating system. The results of this study demonstrated that the system was useful where toxic compounds had similar UV spectra as well as similar retention times.

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In the chromatography of 23 diuretics by Cooper et al. (1989), an HPLC equipped with a diode array UV detector was used. This instrument was linked to a data system. The

extracts were injected onto a Hewlett-Packard Hypersil ODS C18 column or a Hewlett-Packard LiChrosorb RP-18 column. The same gradient mobile phase was used for both columns. The mobile phase Solvent A was a phosphate buffer (pH3) containing propylamine hydrochloride. Solvent B was acetonitrile. Solvent B was increased from 15% at 2min. to 80% at 20min. The diode array detector was set to monitor the signal at 230nm and 275nm.

Diode array detection of 100 basic drugs and metabolites was studied by Logan et al. (1990). The HPLC analysis was performed using a gradient pump system (Varian) operated at 1.5ml/min., with a 10µl loop valve injector. The column was a Hibar Lichrospher 100 CH-8. A UV/VIS diode array detector with a chemstation was also used. The eluent was monitored at 200nm and full spectra were recorded from 190 to 400nm for each peak. The initial mobile phase was composed of 10% acetonitrile in 0.05M, pH 3.2, potassium phosphate buffer. This was increased to 50% over 15min., and the final composition was maintained for 5min. The sensitivity of the diode array detector was less than that of single wavelength instruments. With most of the extractions almost 100% recovery were achieved.

An HPLC gradient elution system for general toxicological screening was developed by Bogusz and Wu (1991). The use of a diode array detector with library software allowed storage of retention parameters,UV spectra, and peak quality parameters. The retention indices of 225 substances were measured using the 1-nitroalkane scale. The 225 substances examined included therapeutic drugs, drugs of abuse and pesticides. The following instruments were used: A quaternary pump, an autosampler and a diode array detector with Chemstation software (all from Hewlett-Packard). A Supersphere 10RP18 column was used with the following elution gradient: acetonitrile-buffer 0-70% over 30 minutes and then 5 minutes at 70%. The buffer used was triethylammonium phosphate 1.0M, pH3.0. All the experiments were performed at ambient temperature. The absorbance at 220nm was monitored as a pilot signal, and the UV spectra were stored.

1-Nitro-octane was added to each sample as indicator of gradient conditions. The RI values of substances were calculated against corresponding 1-nitroalkanes.

Autopsy blood was extracted using direct ethyl acetate extraction at pH4.6, dried under nitrogen, and reconstituted with 50µl methanol. The applied HPLC system proved its usefulness in routine work, enabling identification of substances on the basis of two parameters namely retention index and UV spectra.

The following chromatographic equipment was used in a class-independent drug screen in forensic toxicology using a photodiode array detector described by Drummer et al. (1993). The liquid chromatograph consisted of two constant flow pumps, a gradient mixing chamber, an auto-injector, a system controller and a photodiode array detector (Shimadzu Instruments), with a PC-AD computer. A back-up tape drive, an additional UV detector operating at 230nm, and an integrator/plotter were connected in series after the photodiode array detector. The photodiode array detector operated in a 1-nm band-pass mode monitoring light from 195 to 650nm. The analytical column was a Spherisorb S5 ODS-2 column. The mobile phase consisted of 10% acetonitrile in 10mM potassium phosphate buffer, pH3.1 (pump A), and 60% acetonitrile in 10mM potassium phosphate buffer, pH3.1 (pump B). Gradient conditions were the following: Isocratic at 0% pump B for 1 minute, linear gradient to 50% pump B over 5 minutes isocratic at 50% pump B for 20 minutes, then linear gradient to 100% pump B over 10 minutes, followed by a hold at 100% pump B for 10 minutes. The total run time was 46 minutes and the flow rate 1.0ml/min. The assay was capable of detecting and identifying therapeutic and toxic amounts of a large number of different classes of drugs and has been successfully used to screen over 1000 postmortem blood specimens.

2.2 Extraction

The recovery of the analyte from the available biological sample is one of the crucial steps in analytical work. Various techniques to achieve this are available but each of these techniques has its own advantages and pitfalls.

A popular extraction procedure which has been in use for many years is the liquid-liquid extraction as described by Street (1962). This is used for the extraction of alkaloids, neutral and acidic poisons in human tissue. After initial precipitation of proteins with hot tungstic acid, the principle underlying the procedure is to change the pH of the aqueous phase at different stages of the extraction process in order to separate the different compounds into various fractions which are then applied and separated by paper chromatography. The aim of this procedure is to separate the basic alkaloids, neutrals and weak acids for identification on paper chromatography.

Goldbaum and Domanski (1966) described a method for the extraction of neutral drugs in biological samples. The problems of impurities, mostly neutral fats, were overcome by using hexane in the final extraction step, to get rid of all hexane soluble impurities, including neutral fats. They used gas chromatography for the identification of the neutral drugs by means of relative retention times.

Extraction procedures in chemical toxicology with separation by distillation, solvent extraction and the use of ion-exchange resins of drugs from aqueous solutions were discussed by Tompsett (1968). Among the organic solvents discussed were chloroform, ether, ethyl acetate, benzene, heptane, and some mixtures as well. Most of the drugs examined in this study possessed high extinction UV spectra and an UV spectrophotometer was used to assess the recoveries of the different drugs tested by distillation and solvent-extraction procedures.

The rapid analysis of a large number of urine samples for drugs was reported by Goldbaum et al. (1972). The method utilised gas-liquid chromatography, as well as a

spectrofluorometric procedure in the final analysis of the samples. The isolation procedure used to separate the basic, acidic and neutral drugs was a liquid-liquid extraction procedure. This procedure permitted the isolation of various drugs with a few manipulative steps. By adjusting the urine to pH 9 and extracting with a mixture of 5% isobutanol in chloroform, basic, acid, and neutral drugs were extracted into the organic phase from which basic drugs were back-extracted with 0.1 N H_2SO_4 and acid drugs with 0.75 N KOH leaving the neutral drugs in the organic phase.

An uncomplicated method of analysis for a variety of drugs subject to abuse and accidental overdose was published by Sine et al. (1972). Sample preparation involved serum extraction and aqueous dilution techniques which were followed by gas-liquid chromatographic analysis. For the extraction of acidic drugs, 0.1 ml concentrated HCl was added to 3 ml of the patient's serum and for the neutral extraction 3 ml of 0.1 M phosphate buffer pH7.4. Chloroform was used as extraction solvent. Drug recoveries ranged from 68 % to 100 %.

The rapid separation of urinary acids by HPLC was studied by Molnar and Horvath (1977). The sample was saturated with NaCl and the pH of the supernatant was adjusted to 13. Ethyl acetate was used as organic solvent to remove neutral and basic organic compounds. The aqueous phase was then adjusted with concentrated HCl to pH 1 and the organic acids extracted with ethyl acetate. After evaporation to dryness in a nitrogen stream, the residue was redissolved in 1 M HCl for injection on the HPLC. The high sensitivity and speed of analysis, and the excellent reproducibility suggested that this technique might be useful to obtain metabolic profiles in routine clinical work.

Berry and Grove (1973) made use of a liquid-liquid extraction procedure to extract drugs commonly involved in poisonings. They suggested a back-extraction procedure for stomach contents to get rid of fats, which normally give problems when a direct organic extract is spotted on a TLC plate. However, when employing a back-extraction, care should be taken to see whether neutral drugs remain in the chloroform layer. They

suggested that for neutral drugs a urine sample should be used and only a direct organic extraction should be made. For the identification of the different drugs they made use of thin layer chromatography (TLC) with various spray reagents.

n-Butyl chloride was proposed by Foerster and Mason (1973) as an extractant in drug screening procedures. They suggested that n-butyl chloride be used instead of some of the other solvents commonly used in liquid-liquid extraction e.g. chloroform, ethylene dichloride, ethyl acetate, ether and hexane. They claimed that the final extracts obtained contained little interfering materials and were therefore suitable for analysis by gas chromatography. Three different extraction methods for basic, acidic and neutral drugs were performed with n-butyl chloride.

A column chromatographic extraction technique involving extraction of acids in nonionised form and amines as ion pairs from the same sample was described by Ehrsson and Knapp (1974). The extraction of a biological sample was performed after the pH had been adjusted to pH 3 and 5 M sodium iodide was added to the sample. The mixture was filtered through a 0.5 g Celite 545 column on which the analytes were retained and from which they could then be eluted with water-saturated ethyl acetate. The whole extraction procedure, including the column packing was performed in under 10 minutes. Compounds as diverse as amphetamine and acetylsalicylic acid were recovered in yields close to 90% but the yield for morphine was only 7%.

A procedure for extracting weak acidic, neutral and basic drugs from urine for thin layer chromatography by using an XAD-2 resin was described by Kullberg and Gorodetzky (1974). This extraction procedure was performed on 20 ml urine samples. Different concentrations of the various drugs under investigation were spiked in these urine samples. The percentage extraction was determined by means of radio isotope assay. The adsorption of morphine, amphetamine and phenobarbital from urine to the XAD-2 resin was found to be dependent on the urine pH, urine flow rate through the column and the urine/resin ratio. Acetylsalicylic acid was not efficiently adsorbed. The reason for

this was probably because this compound is too acidic to be appreciably unionised at pH 8.5.

The distribution and recovery of 86 drugs from three different aqueous acidic or alkaline solutions into organic media was studied by Hackett et al. (1975). They also made a special study of the efficiency of the back-extraction process, i.e. the extraction from the organic phase into acidic or alkaline aqueous media. The three different single extraction procedures for neutral, acidic and basic drugs were performed with HCl/chloroform, back-extracted with 0.45 M NaOH, Na_2CO_3/chloroform back-extracted with 1.8 M H_2SO_4, and Na_2CO_3/ether back-extracted with 1.8 M H_2SO_4. The results from this investigation enabled each system to be evaluated according to the efficiency of the drug recovery from either aqueous or organic solutions. They suggested that when urgency is an important factor, such as in cases involving a possible overdose, the slightly lower recoveries from a single extraction would be compensated for by the time saved in multiple extractions and solvent evaporation The use of excess sodium carbonate in the initial steps of the extractions was found to be a reliable method by which a reproducible pH of 10 could be obtained. Ether and chloroform offered advantages over most of the other solvents because they are polar, volatile and largely immiscible with water. The HCl/chloroform proved satisfactory for the extraction of acid drugs while for most basic drugs the Na_2CO_3/ether procedure proved more efficient than the Na_2CO_3/chloroform procedure. The extraction of barbiturates, with the exception of phenobarbital, from the sodium carbonate solution (pH approximately 10) was possible in spite of barbiturates being classified as acidic drugs.

A novel single-step extraction method for TLC identification, which was capable of testing a wide variety of drugs of abuse was described by Kaistha et al. (1975). The technique involved the use of paper loaded with cation-exchange resin. This paper was soaked in 20 - 50 ml of urine. After 60 minutes the paper was rinsed with deionized water. This paper was then put into a screw-capped jar containing ammonium chloride -

ammonia buffer solution (pH 10), and chloroform-isopropanol (3:1). After shaking, the organic phase was transferred to a centrifuge tube with sulphuric acid (0.5%) in methanol. The solvent was then evaporated and the residue redissolved in methanol and spotted on a TLC plate.

A procedure for the fast screening of urine samples was described by Dadisch et al. (1977). The extraction procedure included the use of a high $(NH_4)_2SO_4$ and ethyl acetate concentration (pH 9.5), which enabled the extraction of most of the basic drugs. Back-extraction with 10% tartaric acid and re-extraction at pH 9.5 purified the extract and concentrated the sample up to 75 times.

The use of bonded phase silica as an extraction medium has been reported for a number of drug classes from serum and urine. Ford et al. (1983) demonstrated the applicability of octadecylsilane (ODS) bonded silica for the extraction of acidic drugs from whole blood for screening purposes. They used GC/NPD and GC/MS (mass-spectrometry) for the identification of the compounds extracted. The method reduced the problems associated with co-extracted lipids, required only a small volume of organic solvents and provided acceptable recoveries well into sub-therapeutic ranges for many acidic drugs. Diazomethane, for methylation, was used in the final step.

The recovery of 28 acidic drugs from water and plasma was investigated by Bailey and Kelner (1984) using hexane, diethyl ether, toluene, n-butyl chloride and chloroform as extraction solvents. The drug classes investigated were barbiturates (9), sulphonamides (3), diuretics (4) and other acidic drugs (12). The pH of each sample was adjusted with 1 N HCl to between pH 1 to 2 and the samples were then extracted with the five different solvents. Anhydrous sodium sulphate was used to dehydrate the ether and chloroform extracts after the organic layer was separated from the aqueous phase. The organic phase was evaporated and the resulting extracted residues reconstituted in 2ml 1N NaOH, and then scanned spectrophotometrically from 200 to 400 nm against its

corresponding blank. As drug classes, the barbiturates, sulfonamides and diuretics were best extracted from both water and plasma by diethyl ether.

A one-step clean-up procedure was used by Chan and Chan (1984) in their extraction procedure for acidic and neutral drugs in post-mortem blood. After acidifying the sample, ethyl acetate was used as organic solvent. The organic phase was then dried and the residue reconstituted in methanol. The relative retention times of various drugs were determined by HPLC using a multi-step linear gradient elution program of acetonitrile and phosphtae buffer (10^{-3} M, pH 3.2). The internal standard used was tolylbarbital 5µg/ml. The concentrations of the other drugs were 5µg/ml or 10µg/ml, except for salicylic acid which was 50µg/ml. Forty-eight drugs and metabolites were screened by the authors using this HPLC procedure.

Three isocratic eluents prepared from isopropanol, formic acid and an aqueous phosphate buffer have been used by Stevens and Gill (1986) for the HPLC analysis of analgesic and non-steroidal anti-inflammatory drugs in forensic toxicology. These eluents were used to chromatograph more than 40 analgesic drugs on an ODS silica HPLC column. To whole blood samples was added blood precipitant (diluted perchloric acid containing the internal standard, N-propionyl-p-aminophenol) and the sample vortexed briefly. After centrifugation, 5 µl of the clear supernatant was injected onto the HPLC system.

Salicylic acid and paracetamol together with a wide range of other anti-inflammatory drugs were analysed by Moore and Tebbett (1987). Bond-Elute® solid phase extraction (SPE)columns, containing C8 packing material were used for the extraction. The columns were activated by washing with methanol followed by 0.01M hydrochloric acid. After the blood sample was applied to the column it was washed with distilled water before elution with methanol : 1% aqueous NH_4OH : acetonitrile (50 : 30 : 20). The eluent was evaporated to dryness and the residue was redissolved in methanol. These

samples were then injected onto a HPLC column. To prevent SPE column blockage, blood samples were ultrasonicated before analysis.

A comprehensive screening procedure using HPLC for the detection of 23 diuretics in urine was developed by Cooper et al. (1989). Two aliquots of 2ml urine samples were extracted separately with ethyl acetate under acidic and basic conditions. The acidic and basic extracts were pooled, evaporated to dryness and reconstituted in methanol containing the external standard, ß-hydroxymethyl theophylline. The acidic ethyl acetate extract was shaken with 5% aqueous lead acetate solution to remove urinary pigments and other extraneous materials before combining it with the basic ethyl acetate extract. Gradient elution HPLC was used to separate the extracted components detected with a diode array detector.

A rapid screening method for basic drugs using HPLC and diode array detection was published by Logan et al. (1990). The system involved cation exchange solid-phase extraction for 100 basic drugs and their metabolites, and gradient elution HPLC with diode array detection.. The solid-phase extraction was performed on 1-ml Bond Elute strong cation exchange (SCX) columns. The method was compared with an existing liquid-liquid extraction/capillary GC procedure and was at least as effective for extracting and identifying basic drugs.

A single-column solid-phase extraction procedure was developed for the isolation of acidic, neutral and basic drugs from whole blood by Chen et al. (1992). In this study, the use of Bond Elute Certify mixed-mode SPE columns, which contained both hydrophobic and cation functional groups for the extraction of a broad selection of acidic, neutral and basic drugs from plasma within one run, was investigated. The drugs were eluted in two groups, according to their physical properties and analysed by gas chromatography. The column was preconditioned with methanol, followed by phosphate buffer (pH 6.0). After the sample application, the column was washed with deionized water, and a pH adjustment (acidic) was made. Elution of the different fractions was then made with

acetone : chloroform (1 : 1) followed by 2% ammoniated ethyl acetate. Most of the acidic and neutral drugs were eluted with the acetone : chloroform and the basic drugs were present in the ammonia ethyl acetate eluent. Three different precipitation methods were investigated but the combination of sonication and dilution was found to be the most effective and preferred technique for the pretreatment of whole blood.

Another class-independent drug screening procedure suitable for the use in clinical and forensic toxicology was developed by Drummer et al. (1993). This gradient-elution high-performance liquid chromatography and diode array detection method is capable of detecting and identifying therapeutic and toxic amounts of; barbiturates, anti-convulsants, diuretics, non-steroidal anti-inflammatory drugs, sulphonylurea anti-diabetic drugs, theophylline and analgesic drugs. Standards were prepared in groups representing concentrations likely to be encountered at the high end of the therapeutic range. Samples (blood) and standards were added to polypropylene micro extraction tubes followed by acetonitrile containing p-methylphenyl phenylhydantoin as internal standard to precipitate proteins. The samples were properly mixed and allowed to stand for 10 minutes and then centrifuged at high speed (± 10 000g) for 10 minutes. An aliquot of the supernatant (20µl) was then injected into a HPLC.

At the end of the chromatographic procedure the relative retention times (RRT) of peaks were calculated and the components identified based on their RRT and UV spectra. An advantage of this method was that no selective extraction procedure was used to clean up the blood samples thus allowing any substance, if present in sufficiently high amounts, to be detected.

2.3 Recovery

Except for immunoassays, most analytical toxicology procedures require isolation of the drugs from either an aqueous or biological matrix (usually plasma or urine) prior to the actual measurement. A very important aspect of any isolation procedure is the extent of

the recovery of the analyte from the matrix since this will often determine whether a component will be detected especially when one deals with some of the modern potent drugs. Most published analytical procedures usually contain a report of recovery and in this respect all the extraction procedures mentioned earlier have been reported on in the publications cited. Since no single extraction procedure can be singled out as being the preferred procedure for all drugs, the choice of isolation procedure used is often dictated by parameters such as availability, convenience, speed, etc. In the case of emergency toxicology, for instance, where one often deals with overdoses of drugs, speed of isolation is more often than not, more important than good recovery.

2.4 Relevance of cited literature with the current investigation

From the cited literature it is clear that HPLC-based screening procedures are widely used for a large number of drugs. However, many of the published screening procedures were designed to detect a relatively small range of a specific group of drugs and therefore did not suit our need for a broadly based toxicological screening procedure for acidic, neutral and amphoteric drugs.

Most of the recent studies involving HPLC made use of solid phase extraction procedures while liquid-liquid extraction was extensively reported in earlier publications describing mainly GC and TLC screening techniques. Due to the high cost of solid phase extraction and our favourable experience with liquid-liquid extraction, it was decided to use liquid-liquid extraction in this study and to incorporate a lead acetate washing procedure to remove many of the endogenous pigments, co-extracted from urine, into the organic phase. The lead acetate washing procedure was previously developed and successfully applied to the detection of diuretics in urine.

Of the many chromatographic gradient elution mobile phase systems published, the combination of acetonitrile and tetraethyl ammonium phosphate containing buffer

appeared to be the most generally used, and was therefore chosen for the development of this screening procedure as well.

The general acceptance of the diode array detector as an useful instrument for obtaining UV spectra "on-the-fly" for comparison with spectra stored in a spectral library, using established search algorithms, made the decision to use such a detector an automatic one.

CHAPTER 3

Experimental

3.1 Study Design

In the light of the purpose of this study an isolation procedure yielding a final extract which was clean enough to be analysed on a high-performance liquid chromatograph, was developed. For identification, a diode array detector was used in order to acquire UV spectra of eluting components which would be compared with UV spectra of drugs stored in a spectral library. In addition, it was envisaged that retention time data would be used in conjunction with the spectral data to aid in the unequivocal identification of the eluting components.

Based on the literature survey a liquid-liquid extraction procedure was opted for and diethyl ether and ethyl acetate were chosen as the two extraction solvents to be investigated. Solid phase extraction was not considered as a viable option because of the cost of this procedure.

In addition to the 20 analgesic and non-steroidal anti-inflammatory drugs, 40 drugs belonging to a wide variety of classes were included in this investigation since they were acidic, neutral or amphoteric compounds which could feasibly be extracted under the same conditions. Moreover, the possibility existed that they could have been encountered in the chromatograms during the screening of overdose cases.

Once the screening procedure was set up, actual case studies were expected to contribute additional information to the database. This was especially envisaged with respect to metabolites and other compounds which were not amongst the original compounds selected for investigation at the beginning of the project.

Samples received for toxicological screening were analysed and the resulting UV spectra compared with the spectra stored in the library. When new compounds were identified

in these case studies, their spectra were stored in the library along with the retention time data acquired.

3.2 Equipment

3.2.1 A Hewlett-Packard Model HP1090 liquid chromatograph, equipped with a PV5 gradient solvent delivery system and an autosampler, was used.

3.2.2 The detector was a UV/VIS diode array spectrophotometer detector (Hewlett-Packard 1040). The eluent was monitored at 210 nm and full spectra were recorded from 210 to 600 nm for each peak, making use of the peak-controlled spectral acquisition mode. A spectral library was acquired under local conditions for each compound examined. With the detector, 8 different wavelengths could be followed at the same time till the end of a run. The following diode array detector signal and spectral acquisition parameters were applied.

Signals	A	B	C	D	E	F	G
Sample (nm)							
Wavelength:	210	220	230	254	260	280	320
Bandwidth:	30	30	30	30	30	80	80
Reference							
Wavelength:	380	380	380	380	380	380	380
Bandwidth:	100	100	100	100	100	100	100

Store spectrum: Peak controlled 280 records acquired during run

Threshold: 10 mAU

Peakwidth:	0.300 min.	Sampling interval	2240 ms
Stop time:	30.00 min.	Spectrum range from:	210 nm
Post time:	5.00 min.	To:	600 nm

3.2.3 Data acquisition: Hewlett-Packard Pascal Chemstation consisting of an HP9000/300 computer, an HP 153C disk drive and an HP VDU. The Chemstation software allowed the creation of a spectral library of substances as well as the automatic comparison of experimental data with the library data (retention time and UV spectra).

3.2.4 A Hewlett-Packard Think Jet printer and a model HP 7470A plotter were used for hard copy output.

3.3 Materials

3.3.1 Pure drug substances were obtained from pharmaceutical companies. Stock solutions of these drugs were prepared at a concentration of 1mg/ml in methanol. These methanolic solutions were stored in screw-capped scintillation vials in the refrigerator at a temperature of 2 - 8 °C.

3.3.2 A Phase Sep® S5 ODS1 (15 cm x 4.6 mm) column with a Perisorb® RP-18 guard column was used, both at ambient temperature.

3.3.3 The mobile phase consisted of two component solutions A and B which were pumped and mixed by the solvent delivery system to produce the required mobile phase gradient:

Mobile phase component A (MPCA): 30 g Tetraethylammonium hydroxide (20% solution in water) was diluted with water to about 800 ml, the pH adjusted to 2.8 by the addition of 5.8 g H_3PO_4 and the solution then made up to 1000 ml with water. To 900 ml of this solution, referred to as the TEAP

buffer, 100 ml acetonitrile was added and the resultant solution referred to as MPCA.

Mobile phase component B (MPCB): To 1 litre of acetonitrile, 100µl H_3PO_4 was added.

A mobile phase gradient was used to increase MPCB from 30% at time 0 to 50% at 30 min. (effectively increasing the acetonitrile content of the mobile phase from 40% to 60%). A re-equilibration time of 5 min. was allowed after each gradient run. The flow rate of the mobile phase was kept at 1ml/min. The 10% acetonitrile in MPCA was chosen to prevent micro-organism growth in the TEAP buffer while allowing gradients to be constructed starting with a lower percentage of acetonitrile when required, without having to make up a fresh solution of MPCA.

3.3.4 Lead acetate solution was prepared by dissolving 7.5g lead acetate in 150 ml deionized water.

3.3.5 All solvents used were HPLC grade except diethyl ether which was analytical grade and was distilled before use. The water used, was purified by a Mill-Q filtration system to a resistance above 18 megaohm cm^{-1}.

3.3.6 Internal standard solution A (ISA): 25 mg Flufenamic acid was dissolved in 100 ml of mobile phase consisting of 1 part of MPCA and 1 part of MPCB. One hundred µl of this solution was used in the final reconstitution of the samples analysed.

Internal standard solution B (ISB): 25 mg Carbamazepine was dissolved in 100 ml of mobile phase, consisting of 1 part of MPCA and 1 part of MPCB. One hundred µl of this solution was used as the internal standard in the

recovery of groups 13 and 14 because the retention times of meclofenamic acid and flufenamic acid were identical.

3.3.7 10% H_3PO_4 (112.35 ml of a 89.0% H_3PO_4 was made up to 1 litre).

3.4 Extraction

The stock solutions of the 60 drugs studied in the course of this project, were injected onto the HPLC one at a time, in order to determine their retention times in the system. Retention times were compared during the case studies with the urine of patients. To avoid having to perform the extraction experiment separately on each of the 60 compounds, the drugs were combined into 14 groups of at least 2, and at most 8, components per group. These groups were selected on the basis of their different retention times ensuring good resolution for the different substances.

Two ml drug-free urine samples were spiked for the extraction experiments with the various groups of components at a concentration of 25 µg / 2 ml for each component. This concentration was chosen for analytical convenience as well as to represent toxicologically relevant levels for most of the drugs tested. A reference standard was prepared by spiking 25 µg of each of the drugs in a specific group into an empty glass ampoule. The solvent was then evaporated under a stream of nitrogen. One hundred µl of the mobile phase, containing 60% acetonitrile and the internal standard, was then used to reconstitute the standards to yield a concentration of 2.5 µg / 10 µl of each component in the final injection solution.

The extraction procedure to determine the recovery of each drug are presented in the following table.

EXTRACTION 1		EXTRACTION 2	
1.	2ml urine sample (B14 centrifuge tube)	1.	2ml urine sample (B14 centrifuge tube)
2.	add 500µl 10% H_3PO_4	2.	add 500µl 10% H_3PO_4
3.	add 5ml diethyl ether	3.	add 5ml ethyl acetate
4.	Shake by hand for 2 min.	4.	Shake by hand for 2 min.
5.	Centrifuge for 2 min.	5.	Centrifuge for 2 min.
6.	Transfer diethyl ether into a 10 ml ampoule	6.	Transfer ethyl acetate into a 10 ml ampoule
7.	Add 2ml lead acetate solution	7.	Add 2ml lead acetate solution
8.	Vortex for 2 min.	8.	Vortex for 2 min.
9.	Centrifuge for 2 min.	9.	Centrifuge for 2 min.
10.	Transfer diethyl ether into a 5ml ampoule	10.	Transfer ethyl acetate into a 5ml ampoule
11.	Evaporate at ~40 °C under nitrogen	11.	Evaporate at ~40 °C under nitrogen
12.	Reconstitute in 100 µl mobile phase (60% acetonitrile) containing I.S.	12.	Reconstitute in 100 µl mobile phase (60% acetonitrile) containing I.S.
13.	Inject 10µl on HPLC	13.	Inject 10µl on HPLC

The lead acetate wash was performed to remove urinary pigments and other extraneous material from the extracts. Samples with a recovery lower than 10% were re-analysed without the lead acetate washing step to determine whether the low recovery could be due to the washing step. These samples were spiked in deionized water and extracted with ether. Lead acetate washing had an influence on the recovery of only two drugs, namely paracetamol and sulphacetamide. When the lead acetate wash was omitted, the recoveries for both these substances improved from 0% to ±30%. However, in several of the case studies, paracetamol was found in the urine of patients indicating that this

method can be used to detect paracetamol overdose. The sensitivity of the method varied from compound to compound.

The following table represents the 14 groups of drugs studied, their retention times as well as their relative retention times with respect to flufenamic acid used as internal standard.

The heading "Description" refers to the compound classes: A - Acidic, B - Basic, AM - Amphoteric, and N - Neutral

Group 1		**Retention time (min.)**	**Relative Retention time**	**Description**
1.	Mafenide	1.46	0.06	AM
2.	Paracetamol	2.10	0.09	N
3.	Sulphadimethoxine	4.80	0.21	AM
4.	Cinnamic acid	5.43	0.24	A
5.	Carbamazepine	6.94	0.30	N
6.	Bezafibrate	11.00	0.48	A
7.	Mefenamic acid	21.84	0.95	A

Group 2		Retention time (min.)	Relative Retention time	Description
1.	Sulphasomidine	1.98	0.09	AM
2.	Sulphathiazole	2.33	0.10	AM
3.	Sulphadoxine	3.63	0.16	AM
4.	Furosemide	5.67	0.25	A
5.	Alclofenac	9.02	0.39	A
6.	Sulindac	10.95	0.48	A
7.	Bumadizon	14.94	0.65	A
8.	Ibuprofen	17.28	0.75	A

Group 3		Retention time (min.)	Relative Retention time	Description
1.	Sulphacetamide	2.36	0.10	AM
2.	Salicylic acid	3.84	0.17	A
3.	Tolmetin	9.10	0.39	A
4.	Probenecid	12.02	0.52	A
5.	Flurbiprofen	15.50	0.67	A
6.	Indomethacin	18.12	0.78	A

Group 4		Retention time (min.)	Relative Retention time	Description
1.	Hydrochlorothiazide	2.51	0.11	AM
2.	Sulphamethoxazole	3.79	0.16	AM
3.	Ketoprofen	9.73	0.42	A
4.	Fenbufen	11.77	0.51	A
5.	Diclofenac	17.49	0.76	A

Group 5		Retention time (min.)	Relative Retention time	Description
1.	Sulphapyridine	2.55	0.11	AM
2.	Benzoic acid	3.83	0.17	A
3.	Tiaprofenic acid	9.32	0.40	A

Group 6		Retention time (min.)	Relative Retention time	Description
1.	Sulphamoxole	2.59	0.11	AM
2.	Sulphafurazole	4.02	0.17	AM
3.	Naproxen	10.16	0.44	A

Group 7		Retention time (min.)	Relative Retention time	Description
1.	Acetanilide	3.65	0.16	N
2.	Benzthiazide	7.16	0.31	AM
3.	Bisacodyl	12.30	0.54	B
4.	Glibenclamide	19.70	0.86	A

Group 8		Retention time (min.)	Relative Retention time	Description
1.	Acetazolamide	2.08	0.09	AM
2.	Chlormezanone	5.48	0.24	AM
3.	Chlorpropamide	6.87	0.30	A
4.	Cyclopenthiazide	9.14	0.40	AM

Group 9		Retention time (min.)	Relative Retention time	Description
1.	Caffeine	2.67	0.12	B
2.	Phenolphthalein	6.79	0.30	A
3.	Tolbutamide	8.26	0.36	A

Group 10		Retention time (min.)	Relative Retention time	Description
1.	Phenformin	1.84	0.08	AM
2.	Chloramfenicol	3.76	0.16	A
3.	Chlorzoxazone	5.55	0.24	B
4.	Acetohexamide	8.59	0.37	A

Group 11		Retention time (min.)	Relative Retention time	Description
1.	Theophylline	2.04	0.01	B
2.	Clopamide	4.26	0.19	AM

Group 12		Retention time (min.)	Relative Retention time	Description
1.	Ranitidine	1.63	0.07	AM
2.	Aminophenazone	2.53	0.11	B
3.	Tetracaine	4.31	0.19	B
4.	Piroxicam	7.98	0.35	AM
5.	Warfarin	12.99	0.58	A
6.	Niflumic acid	17.08	0.76	A

Group 13		Retention time (min.)	Relative Retention time	Description
1.	Chlorothiazide	2.29	0.10	AM
2.	Sulphamethoxydiazine	2.30	0.13	AM
3..	Flufenamic acid	22.40	1.00	A

Group 14		Retention time (min.)	Relative Retention time	Description
1.	Sulphamethizole	2.71	0.10	AM
2.	Meclofenamic acid	22.22	1.00	A

3.5 Case Studies

Urine samples received for toxicological screening were extracted using extraction procedure 1 (diethyl ether extraction) described earlier. In the final reconstitution of the sample, before injection onto the HPLC column, the mobile phase containing 60 % acetonitrile without the internal standard, was used. This was done because the software

program used, did not make use of relative retention times in the final analysis of the data that are matched with data in its library. However, the use of an internal standard in the extraction of unknown samples is recommended since relative retention times are more reproducible than absolute retention times and this information could be of use for identification purposes when it is available for comparison with the library data. Moreover, during the recovery studies the peak area of the internal standard was shown to be reproducible enough to be used as an indicator of the quantitative extraction yield. In the event of the unknown sample containing the internal standard, this would probably be noticed because the internal standard peak area would be higher than normal.

CHAPTER 4

Results and Discussion

4.1 Chromatography

The best chromatography results were obtained with a Phase Sep® ODS1 column and mobile phase gradient constructed from TEAP buffer (pH 2.8) and acetonitrile.

The following column and mobile phases were also investigated:

Hewlett-Packard microbore column hypersil ODS 5 µm 100 x 2.1 mm.

Mobile phase 1:

MCPA: To 1 litre of 0.05 M KH_2PO_4, 1 ml formic acid was added.

MCPB: Isopropanol

A mobile phase gradient was used to increase MCPB from 5% at time 0 to 65% at 20 min. A re-equilibration time of 5 min. was allowed after each gradient run. The flow rate of the mobile phase was kept at 0.2 ml/min.

The following problems were encountered with this system:

(a) When the gradient reached 65% isopropanol, the column pressure increased rapidly. This was probably as a result of the increased viscosity or possibly due to precipitation of buffer salts in the mobile phase. This proved to be a problem as some of the tested drugs only eluted at 65% isopropanol.

(b) Because of the viscosity of isopropanol, the HPLC system had difficulty to maintain the specified flow rate.

Mobile phase 2:

MCPA: To 1 litre of 0.025 M KH_2PO_4, 1 ml formic acid was added.

MCPB: To 1 litre of acetonitrile, 1 ml formic acid was added.

A mobile phase gradient was used to increase MCPB from 5% at time 0 to 65% at 20 min. A re-equilibration time of 5 min. was allowed after each gradient run. The flow rate of the mobile phase was kept at 0.2 ml/min.

The following observations were made:

(a) The acetonitrile gave less problems than the isopropanol for maintaining the flow rate at 0.2ml/min.

(b) The peak shapes were not acceptable and peaks tended to tail with poor resolution between some of the substances.

Mobile phase 3:

MCPA: To 1 litre of 0.001 M KH_2PO_4, 1% H_3PO_4 was added to adjust the pH to 3.2

MCPB: To 1 litre of acetonitrile, 100 µl H_3PO_4 was added.

A mobile phase gradient was used to increase MCPB from 30% at time 0 to 60% at 40 min. A re-equilibration time of 5 min. was allowed after each gradient run. The flow rate of the mobile phase was kept at 0.2 ml/min.

The following observations were made:

Although some of the peak shapes improved, the chromatography did not improve significantly.

4.2 Recovery

The recovery of each drug was calculated by dividing the peak area ratio of drug / internal standard of each component after extraction from the urine by the peak area ratio of drug / internal standard obtained with the standard injection solution obtained as described above. Results are presented in table1.

Table 1: RECOVERY PERCENTAGES OF 60 DRUGS SEPARATED ON HPLC. THE EQUIPMENT USED AND PROTOCOL FOLLOWED DURING THE SEPARATION PROCEDURE ARE EXPLAINED IN THE TEXT.

	Drug	% Recovery (diethyl ether)	% Recovery (ethyl acetate)
1.	Acetanelide	89.6	84.8
2.	Acetazolamide	0	*
3.	Acetohexamide	38.8	55.2
4.	Alclofenac	69.8	84.6
5.	Aminophenazone	33.9	*
6.	Benzoic acid	23.8	54.0
7.	Benzthiazide	83.2	76.9
8.	Bezafibrate	60.7	62.8
9.	Bisacodyl	70.3	75.1
10.	Bumadizon	57.5	79.6
11.	Caffeine	0	38.6
12.	Carbamazepine	54.2	73.8
13.	Chloramphenicol	73.1	

14.	Chlormezanone	62.0	94.1
15.	Chlorpropamide	72.5	91.0
16.	Chlorothiazide	31.4	*
17.	Chlorzoxazone	83.0	93.1
18.	Cinnamic acid	41.6	58.9
19.	Clopamide	17.2	*
20.	Cyclopenthiazide	77.3	85.2
21.	Diclofenac	63.9	70.0
22.	Fenbufen	66.2	81.4
23.	Flufenamic acid	67.7	82.5
24.	Flurbiprofen	75.9	80.3
25.	Furosemide	20.1	68.5
26.	Glibenclamide	81.9	77.9
27.	Hydrochlorothiazide	17.3	*
28.	Ibuprofen	81.3`	84.2
29.	Indomethacin	71.8	80.3
30.	Ketoprofen	67.6	83.2
31.	Mafenide	0	0
32.	Meclofenamic acid	64.0	66.7
33.	Mefenamic acid	72.6	74.5
34.	Naproxen	74.9	82.0
35.	Niflumic acid	91.4	80.7
36.	Paracetamol	0	*
37.	Phenformin	0	*
38.	Phenolphthalein	84.3	45.5
39.	Piroxicam	81.4	87.0

40.	Probenecid	61.8	87.0
41.	Ranitidine	0	*
42.	Salicylic acid	15.5	48.7
43.	Sulindac	31.9	74.3
44.	Sulphacetamide	0	0
45.	Sulphadimethoxine	64.5	83.9
46.	Sulphadoxine	38.4	97.6
47.	Sulphafurazole	35.4	86.2
48.	Sulphamethizole	4.6	*
49.	Sulphamethoxazole	58.8	98.7
50.	Sulphamethoxydiazine	0	*
51.	Sulphamoxole	0	*
52.	Sulphapyridine	0	*
53.	Sulphasomidine	0	0
54.	Sulphathiazole	0	0
55.	Tetracaine	31.8	55.2
56.	Theophylline	0	*
57.	Tiaprofenic acid	78.7	84.0
58.	Tolbutamide	76.4	42.4
59.	Tolmetin	40.2	74.9
60.	Warfarin	91.7	91.2

* Indicates that the ethyl acetate extracted background interference is too high to be able to identify the drug peak satisfactorily and to calculate the % recovery.

The results in Table 1 indicate that, for most of the acidic drugs, diethyl ether as organic extraction solvent gave good recoveries. Ethyl acetate yielded better recovery results for the sulphonamide group. However, the latter proved to be problematic, because the high

background of endogenous peaks, found in normal urine, often interfere with these low retention time compounds. Washing of the extracts with lead acetate solution was found to be beneficial in so far as concentrations of these endogenous compounds were often reduced in the extracts without the yield of the drugs studied being adversely affected. On the basis of the results of the recovery studies, diethyl ether extraction followed by lead acetate washing of the extract was adopted as the standard isolation procedure for investigating all samples sent in for toxicological screening by HPLC in all the case studies reported in this project..

The chromatograms of blank urines and of the 14 drug groups analysed are presented numerically in Appendix I and the UV spectra of the 60 substances alphabetically in Appendix II. UV Spectrophotometric absorption data are tabulated numerically in Appendix III, and alphabetically in ascending order of peak maximas in Appendix IV.

4.3 Case Studies

In the discussions of the case reports which follow, the UV spectra of the unknowns are drawn in black while the matching spectra from the spectral library are drawn in red. The matched compound's name and its retention time as recorded in the spectral library data base are printed in red and the match quality, expressed as a percentage, is also indicated in each figure.

Case 1

A urine sample and plasma were received for toxicological screening after a 23 year old male patient was admitted to the casualty department with a suspected overdose of paracetamol and tricyclic antidepressants (TAD). Plasma paracetamol and TAD levels were requested and gave the following results: no paracetamol could be found while the

TAD level was 270 ng/ml. These tests were performed using fluorescence polarization immuno-assay (TDX).

An extract of the urine was subjected to HPLC and Fig. 1 represents the chromatogram of the sample analysed. The two major peaks at 4.36 min. and 9.61 min. with spectral matches illustrated in figures 1a and 1b were both identified as naproxen (match qualities of 99.3 and 99.9% respectively) using the library search function. However, only the peak with retention time of 9.61 minutes matched the retention time of naproxen and was therefore identified as naproxen. The good spectral match with naproxen, of the peak with retention time of 4.36 minutes, indicates that this component was probably a metabolite of naproxen. The data for this peak were therefore entered provisionally for future reference as naproxen metabolite data into the spectral library; information which can be corroborated by performing an administration study with naproxen at some convenient time, or by observing whether the same component is again present in the next case involving naproxen. This type of additional metabolite pattern data increases the identifying power of any drug screening procedure considerably and represents the most compelling reason for a toxicology unit to set up its own spectral library for screening purposes.

Paracetamol could not be found in this urine sample. The presence of naproxen and the absence of paracetamol in the sample underlines the unreliability of information often volunteered by patients treated for intentional drug overdoses in the casualty room.

FIG 1

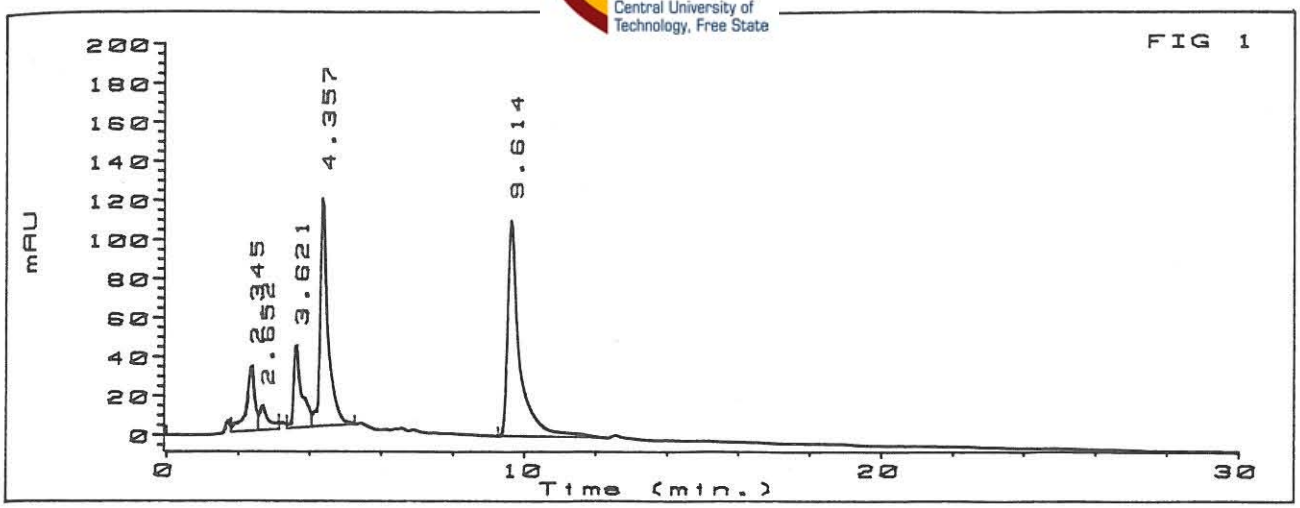


FIG 1a

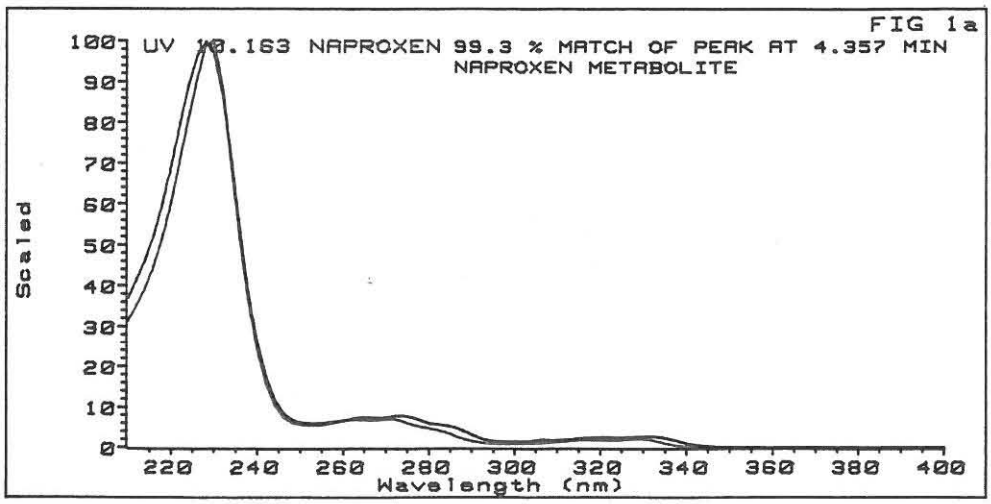
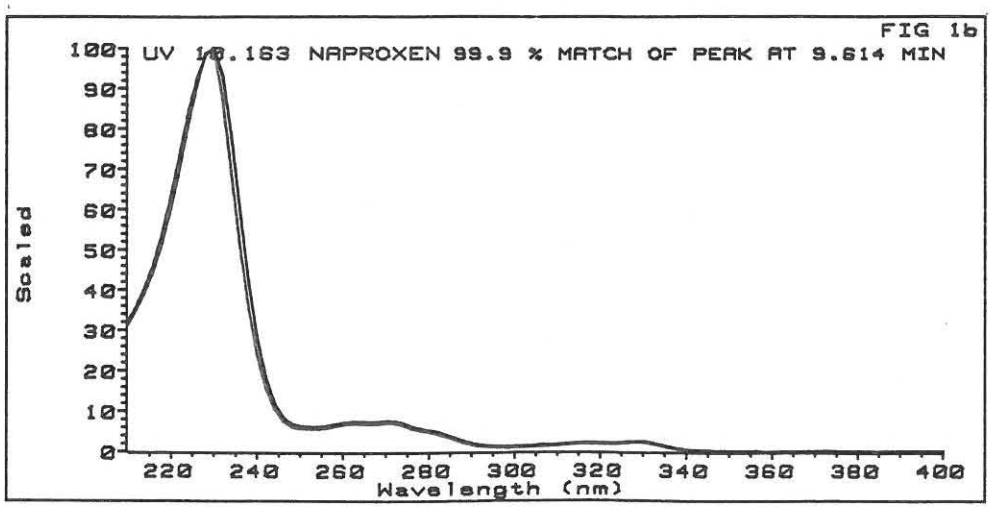


FIG 1b



Case 2

This case represents the data obtained from a 27 year old female patient who had apparently taken an overdose of Tegretol® (carbamazepine) and Brufen® (Ibuprofen). Plasma and urine were received for toxicological screening. A carbamazepine plasma level of 13.0 µg/ml was determined by TDX..

Seven peaks were observed in the chromatogram of the urine extract (fig. 2). Of these, no less than four displayed very similar UV spectra (fig. 2a - 2d) and all matched the spectrum of ibuprofen very closely while only the peak with the retention time of 16.77 minutes matched the retention time of ibuprofen. Just as in case 1, this component was therefore identified as ibuprofen while the other four components were again provisionally added to the spectral library as ibuprofen metabolites. The relative retention times of these four components with respect to ibuprofen were 0.243, 0.265, 0.306, and 0.371. That these were indeed metabolites of ibuprofen was corroborated in another case involving ibuprofen (case 13) in which peaks with relative retention times of 0.241, 0.265, and 0.307 with respect to ibuprofen were also observed. The absence of the peak with relative retention time of 0.371 in case 13, indicates differences in the metabolite patterns between subjects which are not uncommon.

The peak with retention time of 6.92 minutes was identified as carbamazepine on the basis of the perfect spectral match illustrated in fig. 2e as well as on the basis of matching retention times.

FIG 2

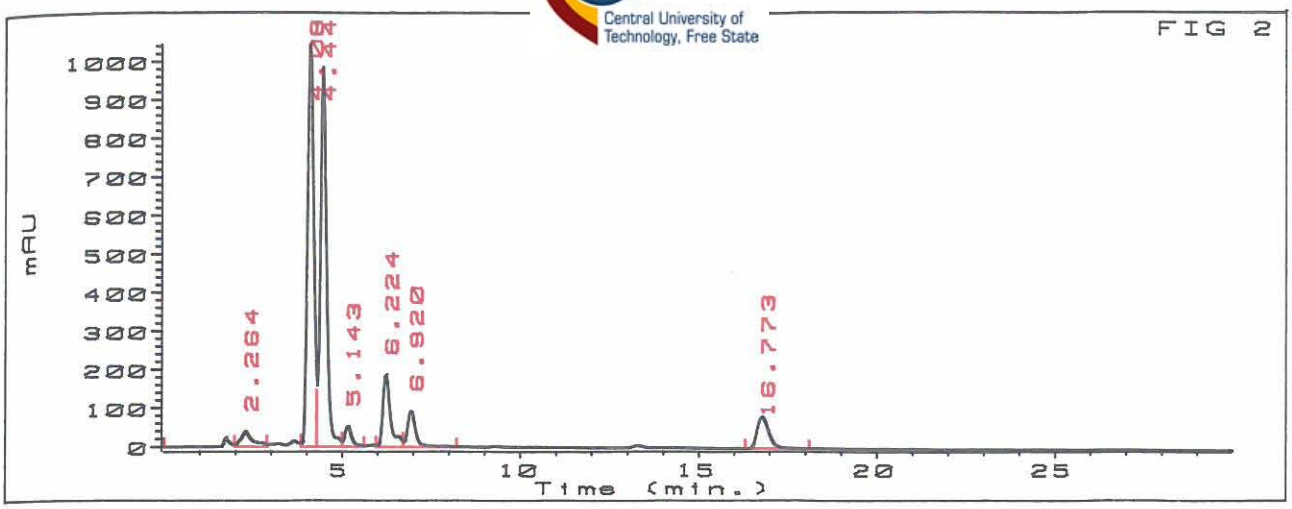


FIG 2a

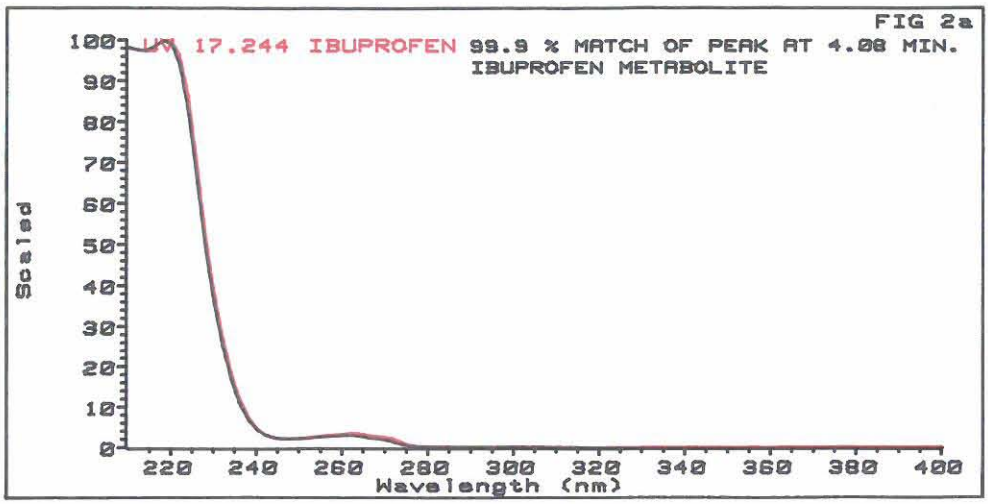
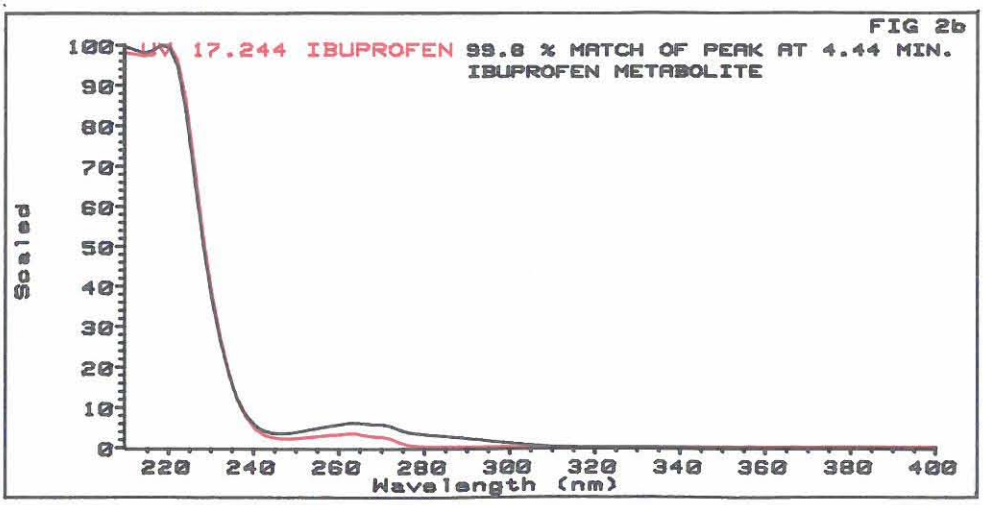
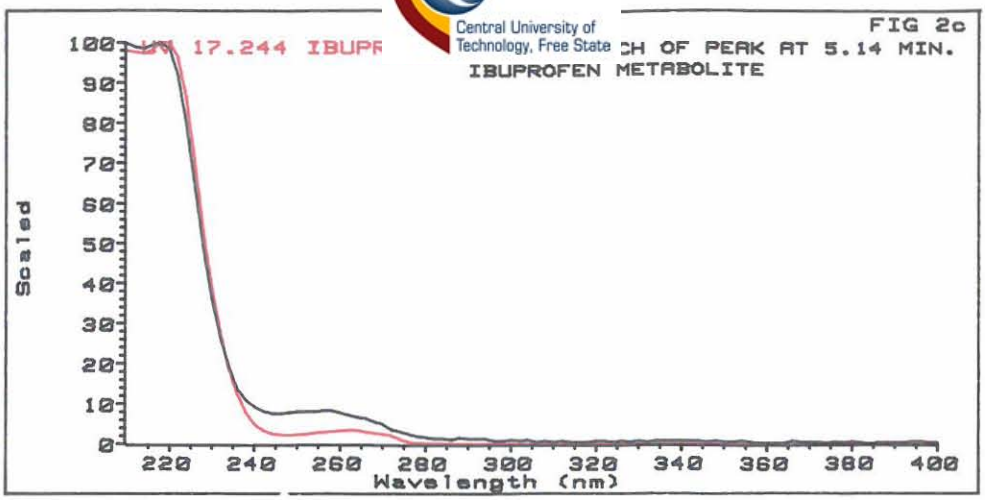
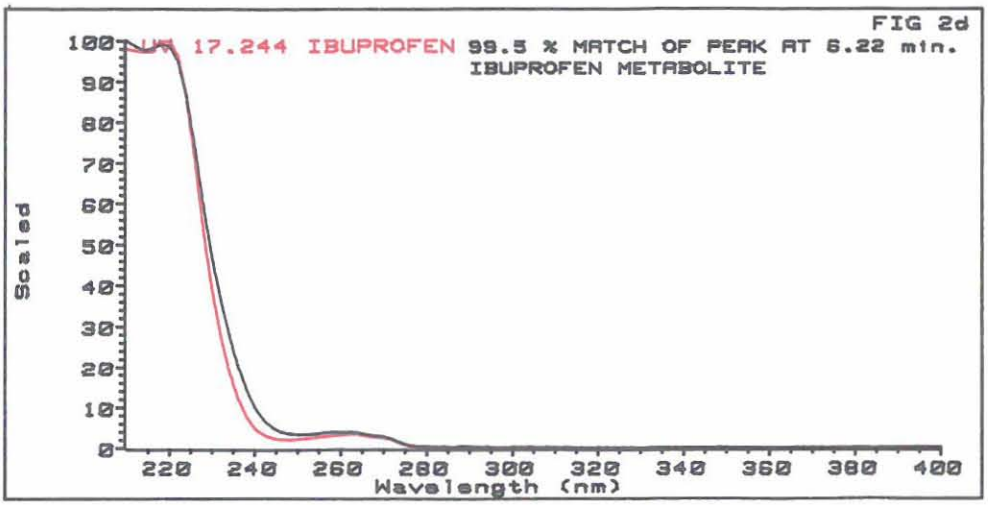
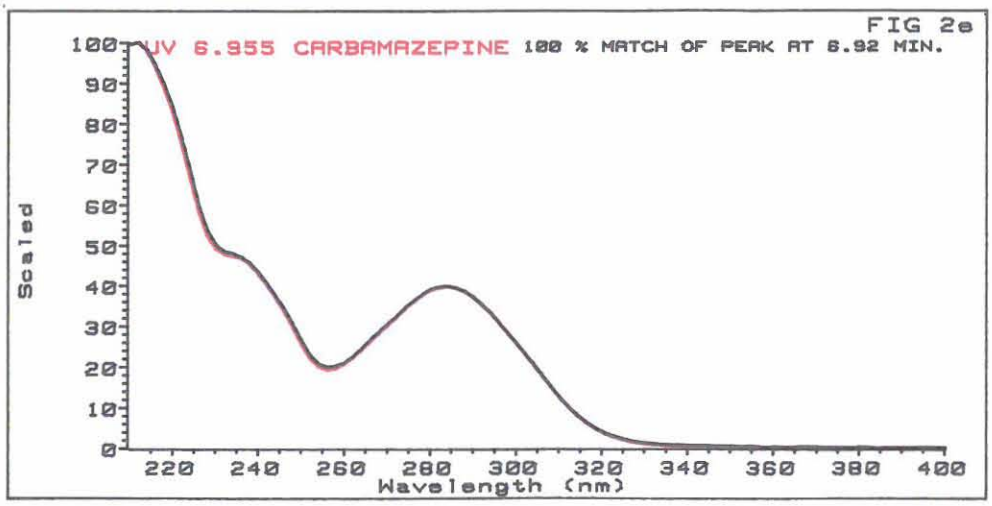


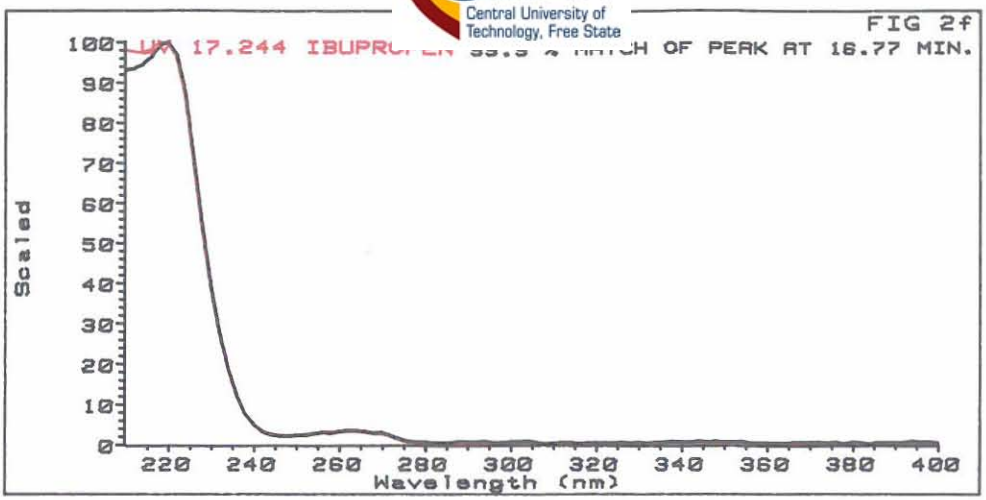
FIG 2b











Case 3

This sample was collected from a 44 year old comatose female patient with the history of a benzodiazepine and alcohol overdose. Although blood and urine were sent to the laboratory, the clinicians only requested plasma benzodiazepine and ethanol levels. The benzodiazepine level was 2114 ng/ml and the ethanol level 190 mg %, which indicated a severe poisoning. The urine sample was also extracted and subjected to HPLC / UV-spectral screening on the next day and the chromatogram (fig. 3) obtained showed two peaks at 2.45 min. and 6.78 min. which were identified as hydrochlorothiazide (fig. 3a, 99.9 % match) and carbamazepine (fig. 3b, 99.7 % match). These results once again stressed the importance of performing a thorough general toxicological screening even when the cause of the patient's condition could apparently be explained on the basis of results already at hand. This is especially so when a patient is comatose since the treatment could be profoundly influenced by the presence of some other drugs which may not have been identified.

FIG 3

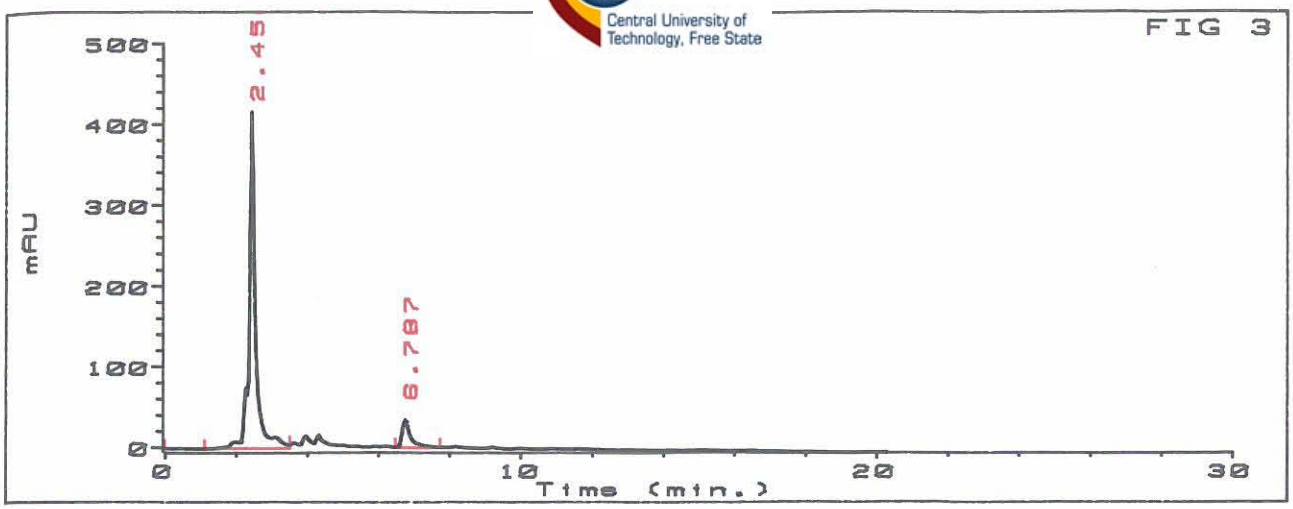


FIG 3a

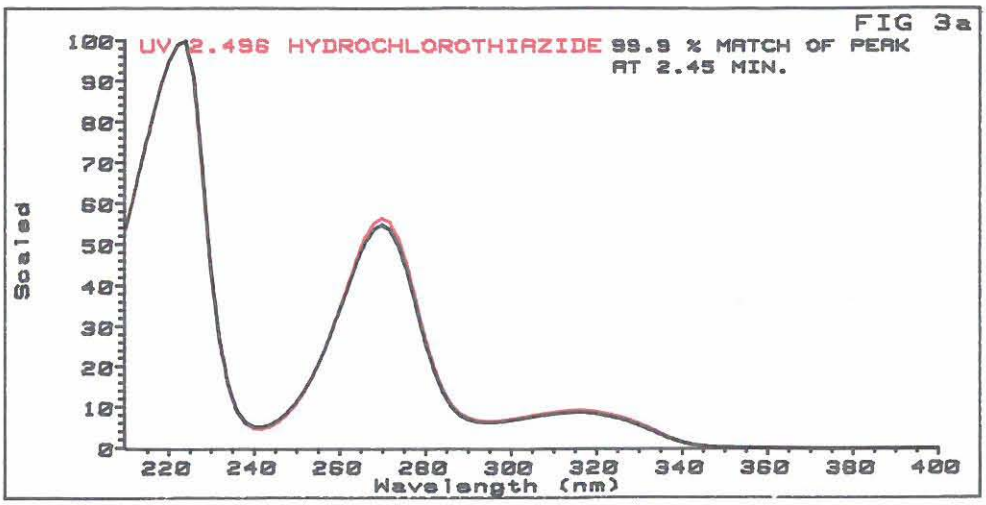
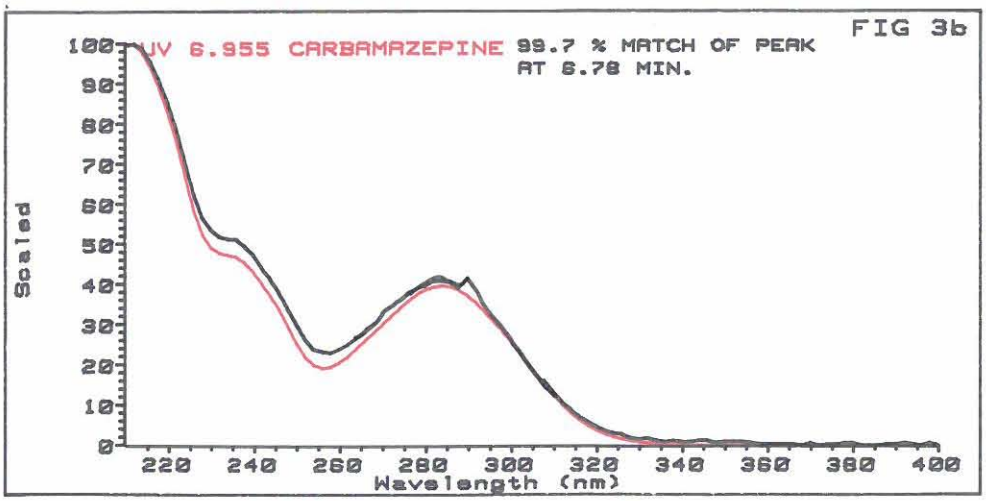


FIG 3b



Case 4

A severe paracetamol overdose case was admitted to the hospital. This 21 year old female patient had reputedly ingested 100 Paraded® (paracetamol) tablets (500 mg) approximately 4 hours before she was admitted. The paracetamol plasma level analysed on the TDX gave a level of 332 µg/ml. The chromatogram obtained when the extract of a urine sample was injected on the HPLC gave a very high absorbance (greater than 2000 mAU) for a peak with a retention time of 2.07 min. in spite of the fact that paracetamol is extracted with a very low yield as shown in the table of recoveries presented in chapter 3. Because no UV-spectra could be obtained as result of the high absorbance, the sample was diluted 4 times (300 µl 60% mobile phase was added to the 100µl that is normally used to dissolve the sample in), and re-injected. Fig. 4 is the chromatogram obtained with the second injection after the sample was diluted. Only one peak with a retention time of 2.07 min. and an absorbance of 800 mAU at 210 nm was observed. A 100% match was found for paracetamol as indicated in fig. 4a. This, together with the correct retention time, confirmed the result of the plasma level analysed on the TDX and proves that paracetamol overdoses can be easily detected using this procedure notwithstanding the poor recovery with the extraction procedure.

FIG 4

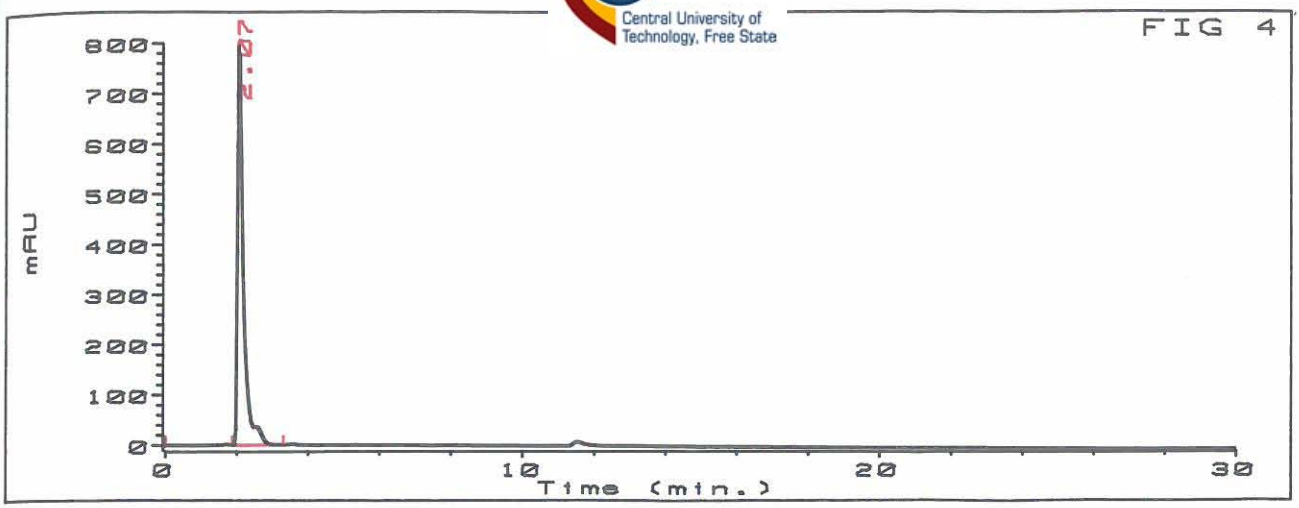
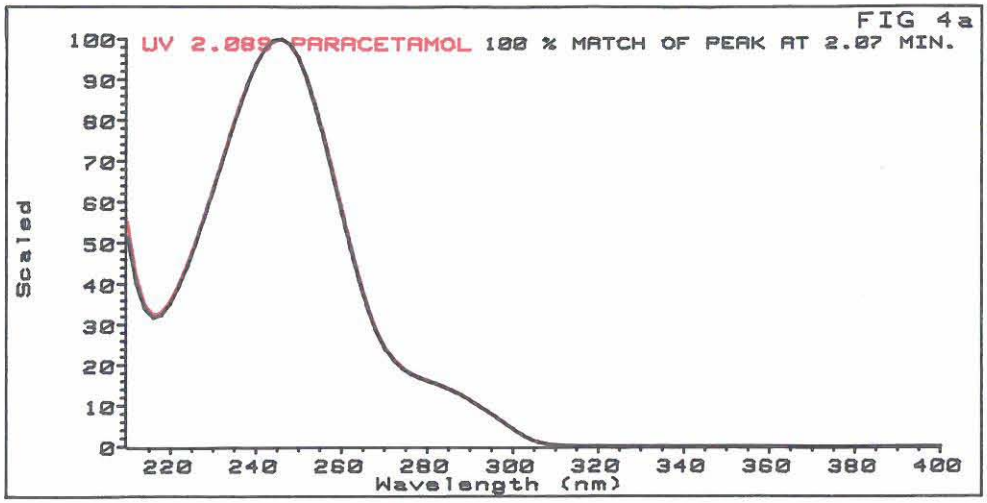


FIG 4a



Case 5

A 24 year old female patient, with a history of a massive overdose of a mixture of medications, died shortly after arrival at the hospital. She left a letter, to indicate that she had taken the following medications: 100 Betapyn® (450 mg paracetamol, 8 mg codeine phosphate), 100 Stemetil® (5 mg prochlorperazine), 70 Voltaren® ,(25/50 mg diclofenac sodium), 30 Panado® (500 mg paracetamol) and 20 Prozac® (20 mg fluoxetine HCl) tablets. The deceased, who was apparently a nursing sister, also indicated that she had injected herself with 10 ampoules Avafortan®.

Urine, blood and stomach contents were collected and sent to the laboratory for toxicological screening. Fig. 5 represents the chromatogram obtained with the extract of the urine sample which was analysed on the HPLC. Two major peaks could be observed on this chromatogram with retention times of 2.09 and 3.70 min. The strong absorbance of the two components indicated that a high concentration of these compounds could be present. The library search for the peak at 2.09 min. revealed a 100% match for paracetamol (fig. 5a). The peak at 3.70 min.. gave a good match of 99% for salicylic acid (fig. 5b) although the peak maxima of the UV spectra differed quite substantially. The same phenomenon, which would be worth investigating more closely at some stage, was observed for salicylic acid on several other occasions. Plasma levels of both drugs were analysed using the TDX analyser and both were confirmed with concentrations of 326 µg/ml for paracetamol and 678 µg/ml for salicylic acid.

FIG 5

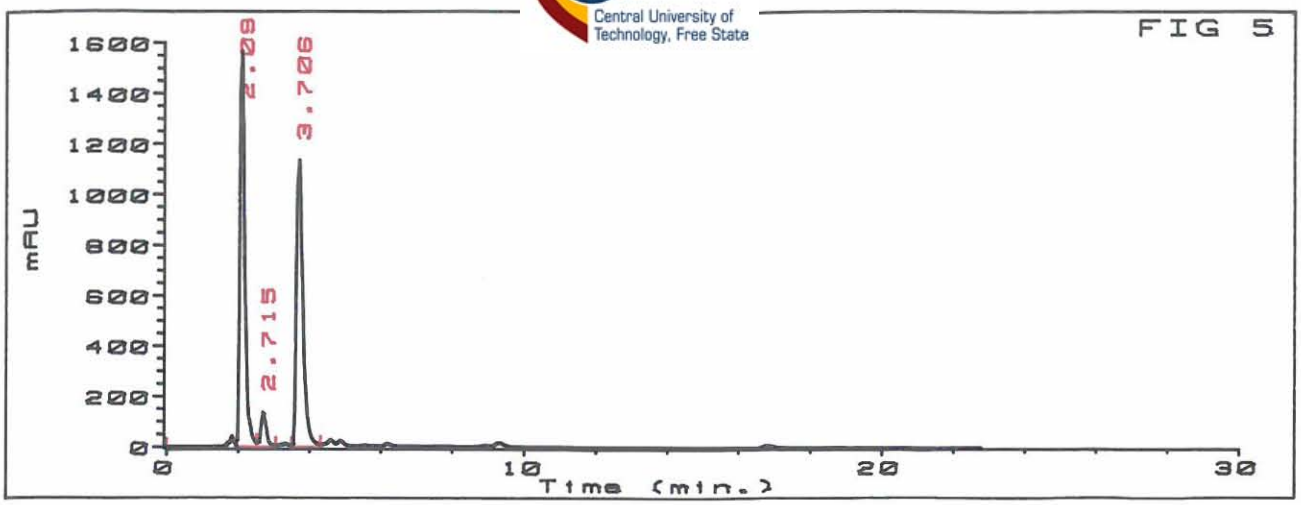


FIG 5a

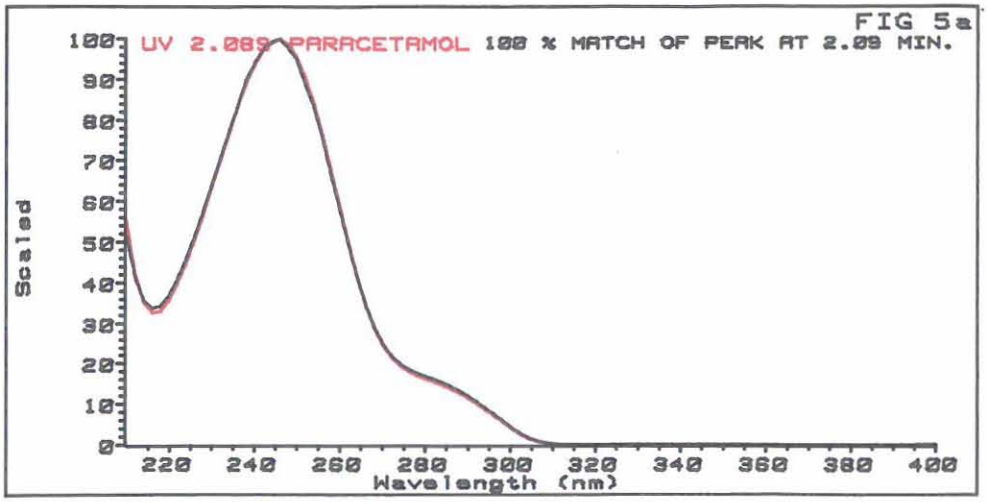
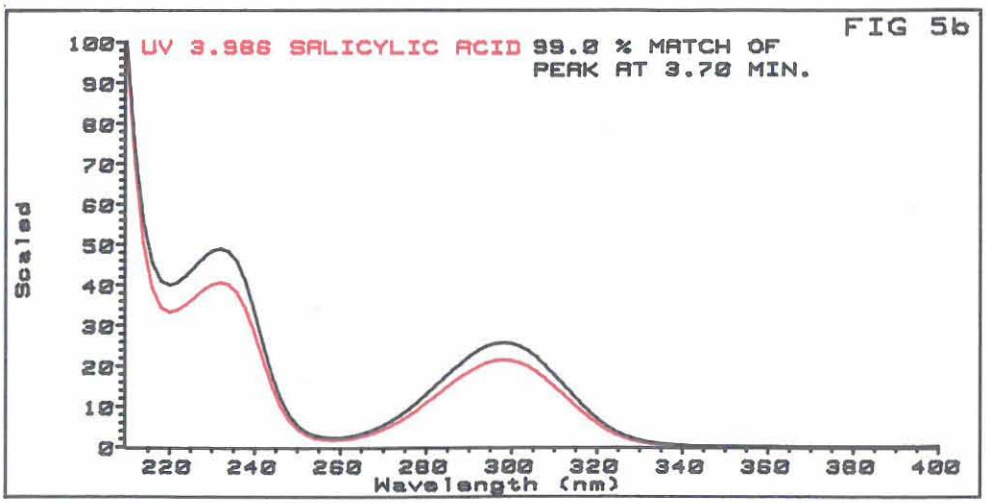


FIG 5b



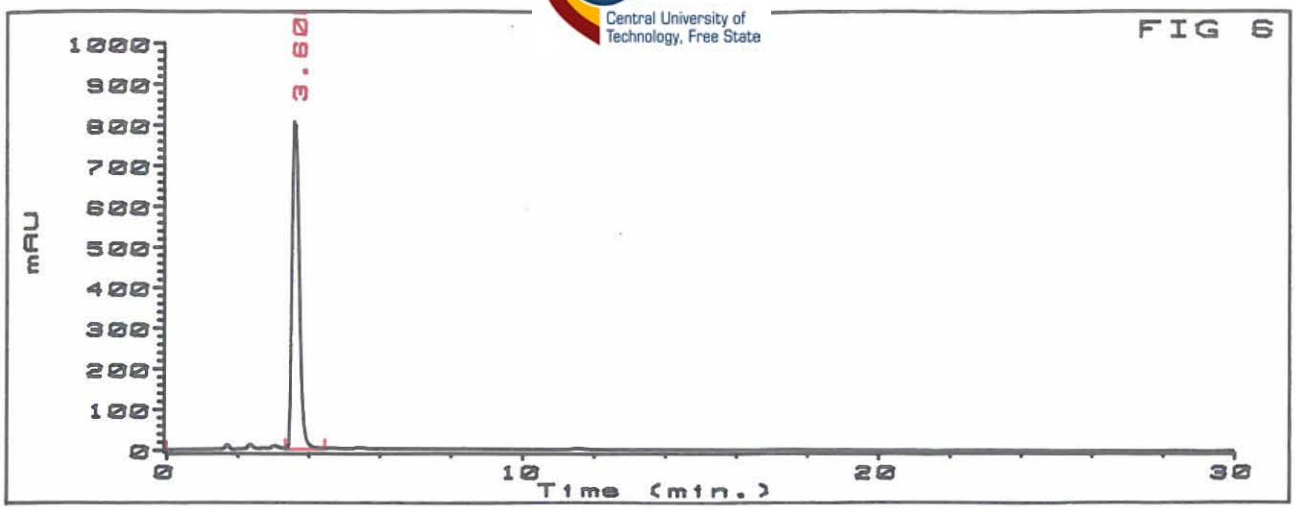
Case 6

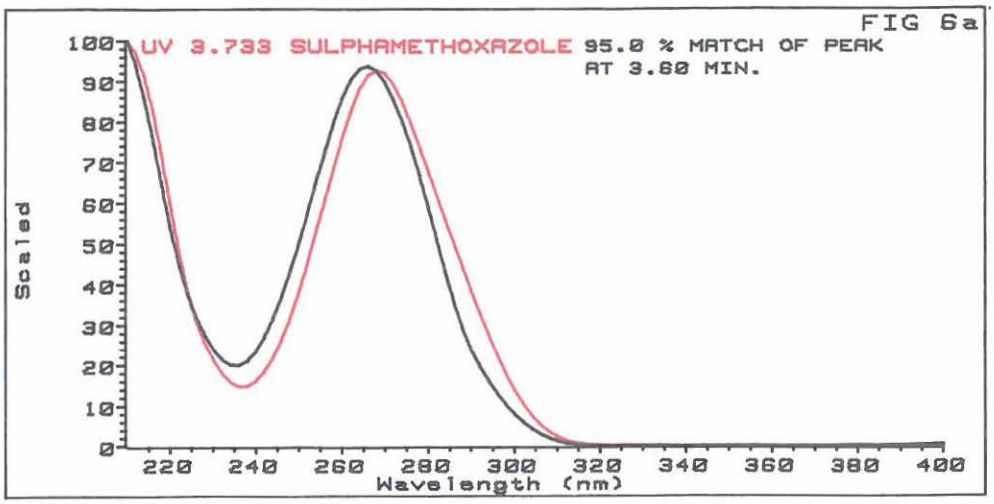
A urine sample, analysed by gas chromatography for the presence of drugs of abuse revealed the presence of trimethoprim. The presence of trimethoprim, which is normally found in combination with sulphamethoxazole led to the analysis of the sample on the HPLC in order to determine whether this sulphonamide drug was indeed present in the urine sample.

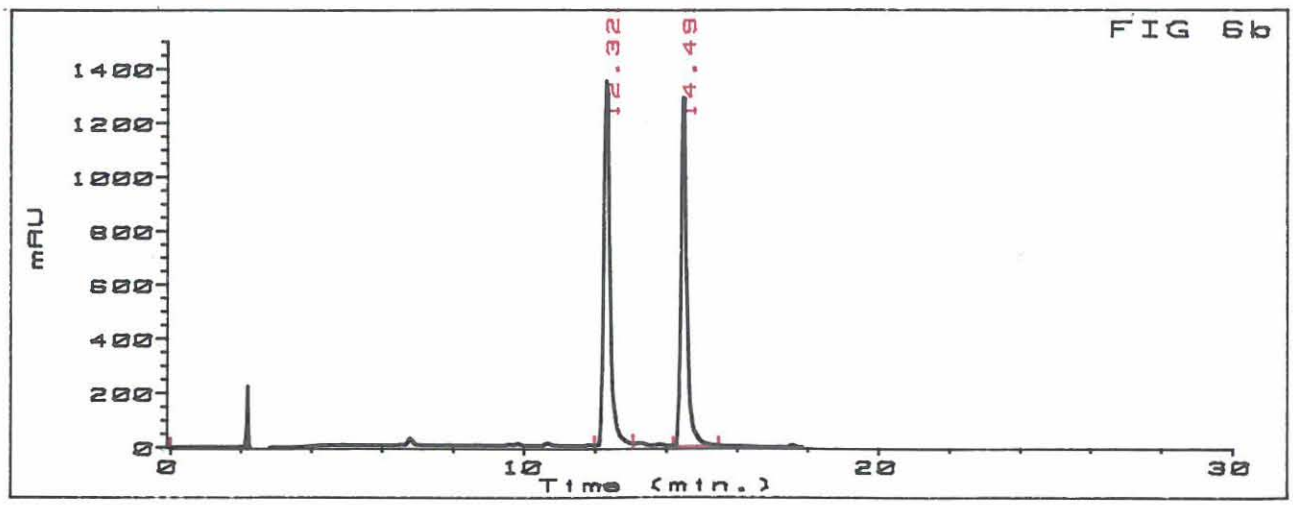
One peak with a retention time of 3.60 min. was present in the HPLC chromatogram (fig. 6) of the urine extract. The library search of the compound gave a 95.0% (fig. 6a) match for sulphamethoxazole. Although the retention time of the peak correlated well with that of sulphamethoxazole, the UV spectra did not give a match as well as one would have expected. In the light of the poor match it was decided to investigate the possibility that our pure substance, which was used as reference standard and of which the spectrum was stored in the library, could have changed during storage. A solution of sulphamethoxazole, prepared from a tablet of Bactrim® and analysed on the system gave a peak with a retention time of 3.76 min., which matched sulphamethoxazole 100% indicating that the spectrum stored in the library was correct. Another possibility was that the peak at 3.60 min. in the sample could contain an impurity or a metabolite of sulphamethoxazole co-eluting with sulphamethoxazole. Using the purity check facility of the diode array detector software, the peak was shown to be impure.

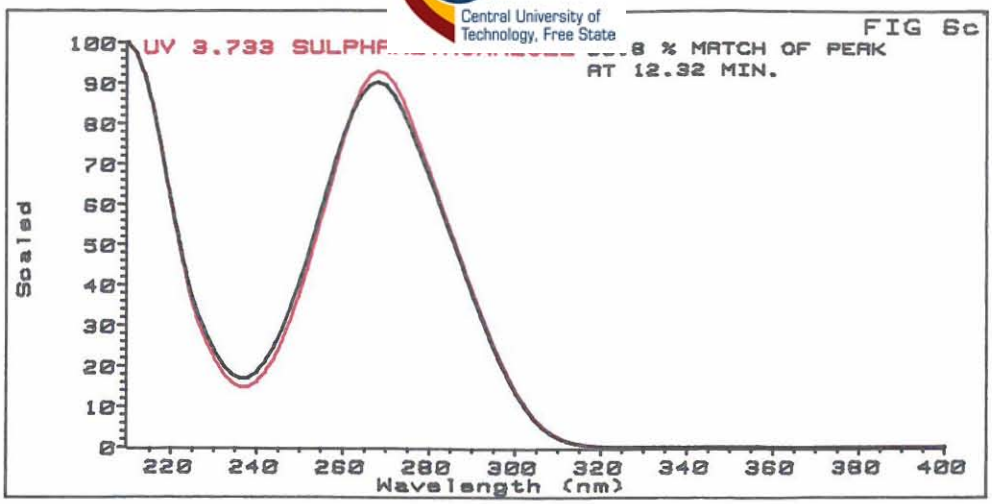
Thus, to investigate the possibility of co-eluting peaks, it was decided to change the mobile phase gradient to see whether a separation of such substances could be effected. The gradient used, was MPCB from 0% to 50% over a period of 30 minutes (effectively increasing the acetonitrile content of the mobile phase from 10% to 60% in this time period compared with the standard change from 40% to 60% over the same time period). The flow rate and all other conditions remained the same as for previous samples analysed. Fig. 6b represents the chromatogram obtained when the sample was run on the new mobile phase gradient. Two major peaks with retention times of 12.32

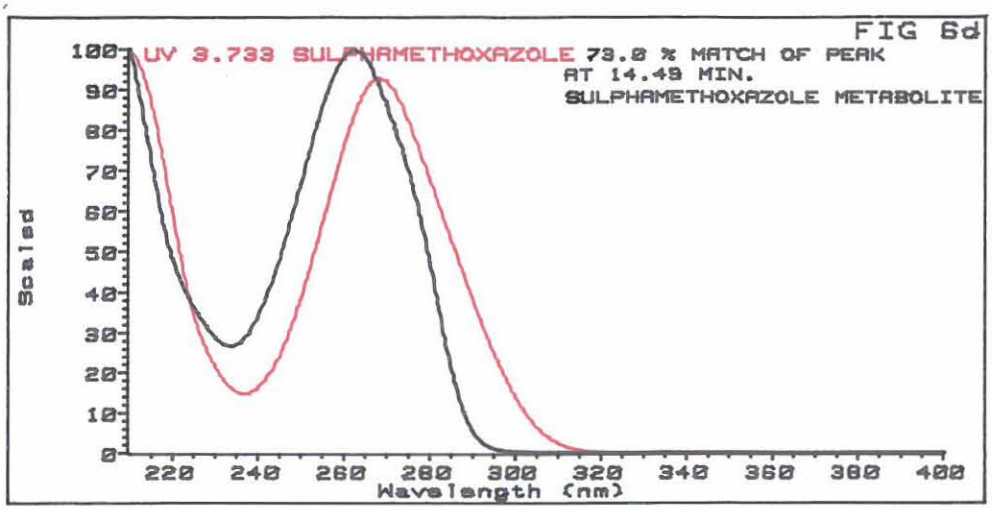
and 14.49 minutes were obtained. This chromatogram clearly demonstrated that two peaks was co-eluting at 3.60 min. in the previous chromatogram (fig. 6). The UV-spectrum of the peak at 12.32 min. now gave a 99.8% match for sulphamethoxazole (fig. 6c) while the peak at 14.49 min. gave a 73.0% match for sulphamethoxazole (fig. 6d). The similarity of the UV spectra suggested that this second peak could have been a metabolite of sulphamethoxazole and the data were therefore stored as such for future reference in the spectral library. This assignment was corroborated by the presence of the same pair of peaks in case report 9.











Case 7

A 17 year old female patient had taken an overdose of Aspirin® (acetylsalicylic acid), approximately 5 hours before urine and plasma were collected for analyses. A plasma level of 229 µg/ml was found for salicylic acid on the TDX. Fig. 7 represents the chromatogram of the extract obtained by HPLC. Fig. 7a presents the match found (100%) for one of the metabolites of acetylsalicylic acid, namely salicyluric acid at 2.71 min. and a 99.4% match was found for salicylic acid, 3.62 min. (fig. 7b). The retention times also correlated well with the retention time of these two compounds.

FIG 7

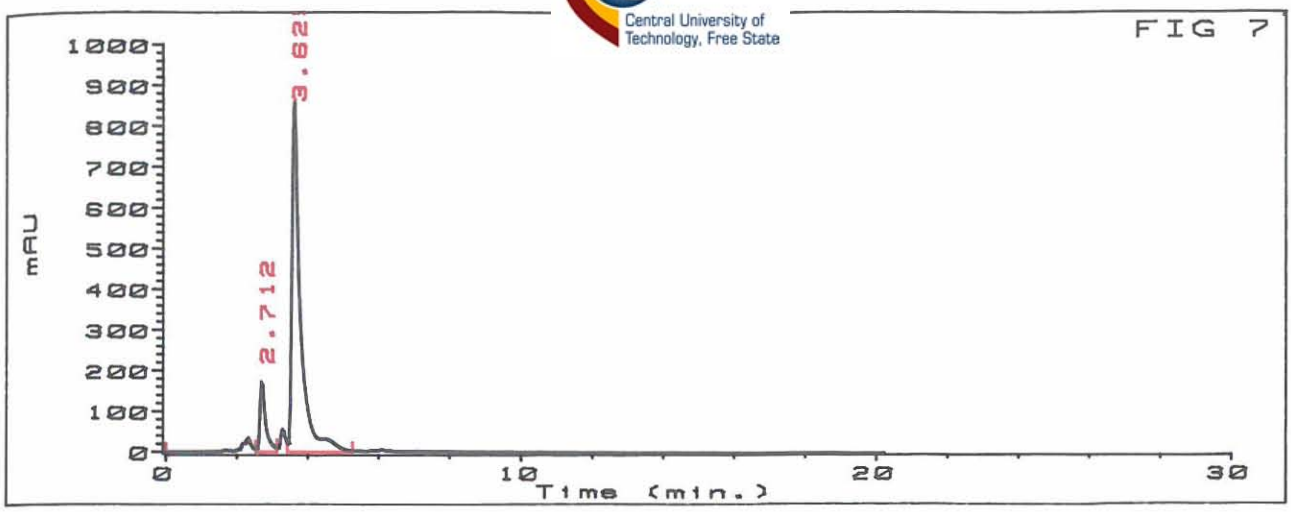


FIG 7a

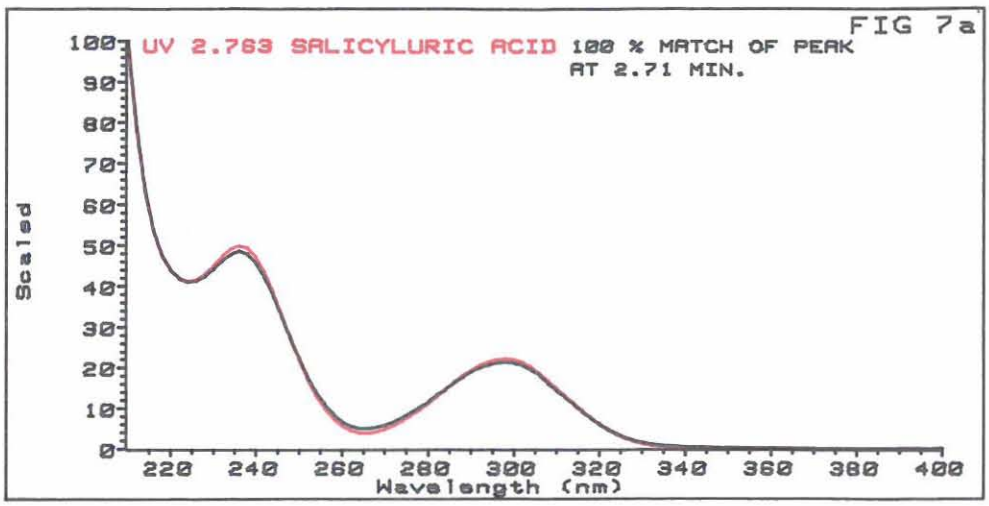
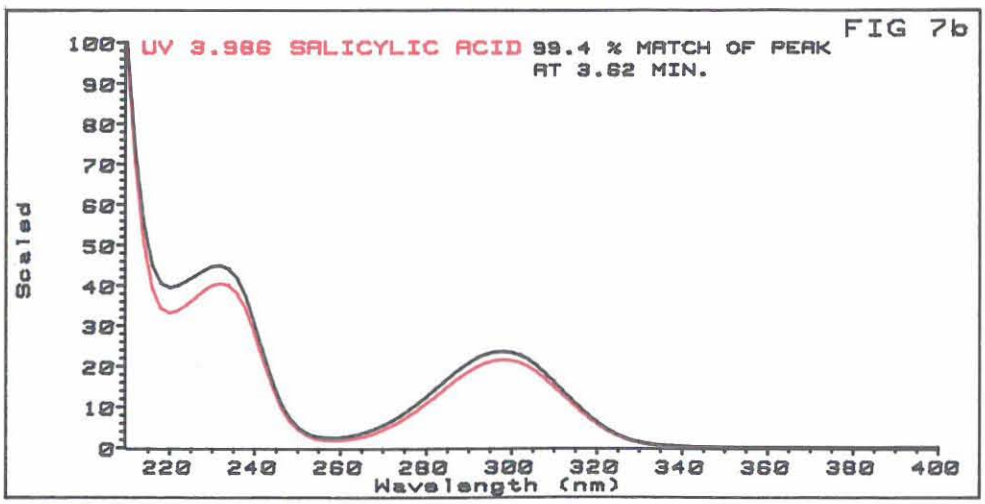


FIG 7b



Case 8

Urine from a 28 year old female patient was sent to the laboratory for toxicological screening. Gas chromatographic screening for basic drugs revealed meprobamate and codeine. Since these two drugs could be found in combination with other analgesics, an HPLC-screening was also performed on the urine sample. The chromatogram obtained (fig. 8) indicated that only small peaks were present of which the two peaks at 2.09 and 5.40 min. gave proper UV-spectra. A 99.5% match for paracetamol (peak at 2.09 min.) and a 99.9% match for furosemide (peak at 5.40 min.) were found, once again emphasising the utility of using a broadly based screening procedure rather than the narrow target compound analyses which are presently so commonly carried out in laboratories which claim to be toxicology laboratories.

FIG 8

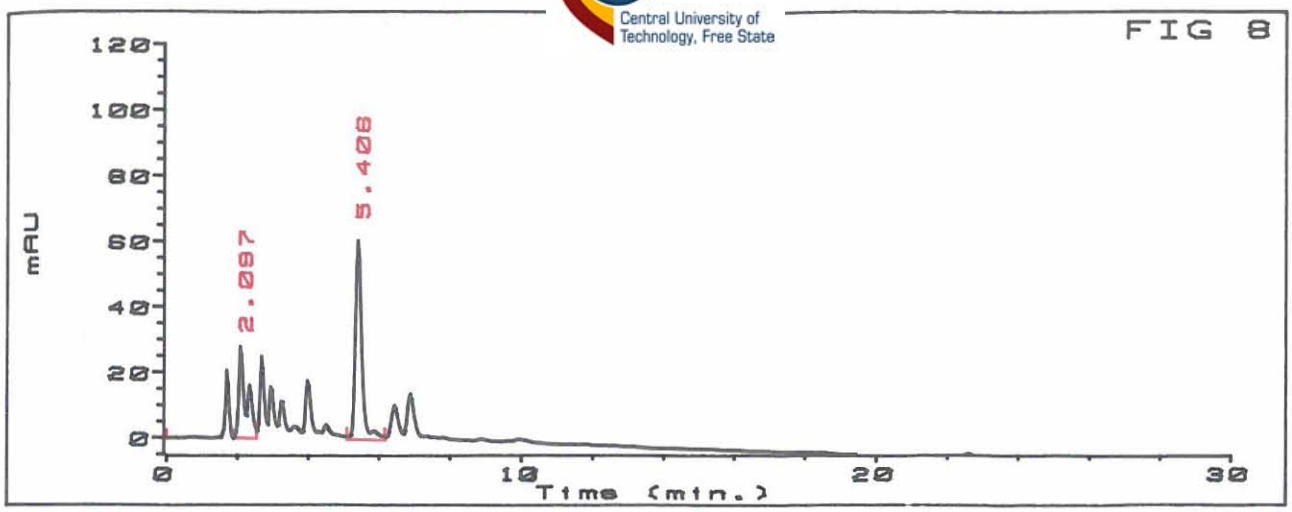


FIG 8a

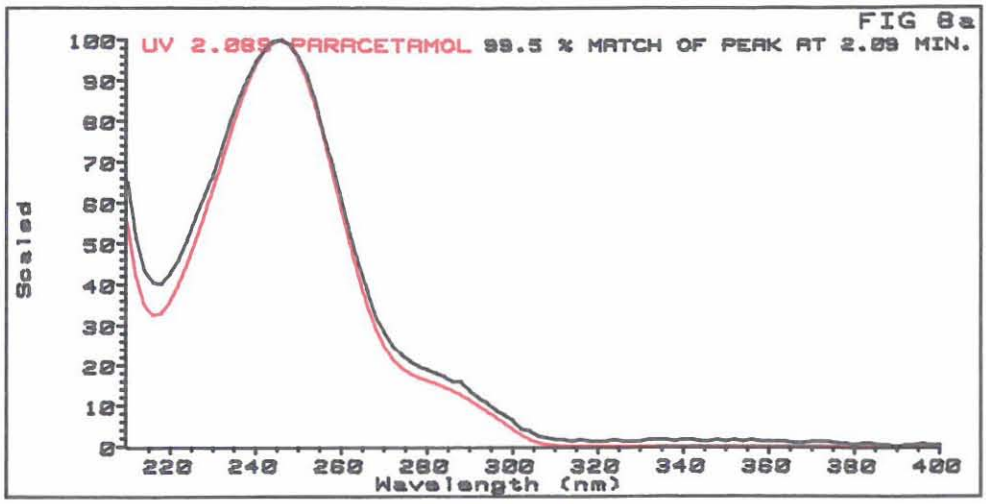
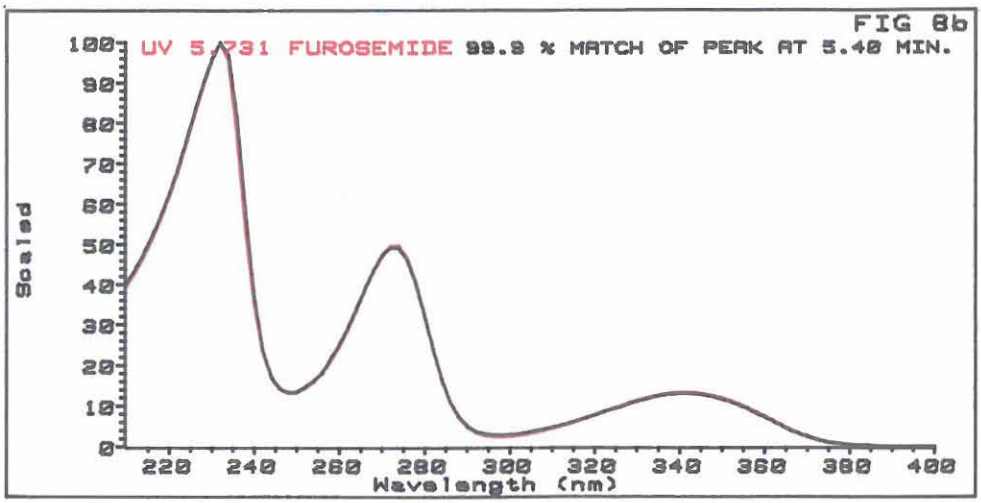


FIG 8b



Case 9

A patient with a suspected overdose of analgesics and antibiotics was admitted to the casualty department. This 21 year old male patient took the medication approximately 6 hours before the sample was collected.

With the initial chromatogram that was obtained, the peak with retention time of 3.69 min. gave an absorbance reading above 2000 mAU at 210 nm. This high absorbance also had the effect that a proper UV-spectrum for this peak could not be obtained. The sample was therefore diluted three times (200µl of a 60% mobile phase was added to 100µl in which the sample are normally dissolved). Fig. 9 represents the chromatogram after the sample was re-injected. Four peaks gave acceptable UV-spectra with which spectral library searches could be made. The peak at 2.08 min. gave a 100% match for paracetamol (fig. 9a). The peak at 2.73 gave a good match of 99.8% for salicyluric acid (fig. 9b).

The next two peaks at 3.11 and 3.69 min. indicated the possible presence of sulphamethoxazole with matches of 98.0% (fig. 9c) and 91.0% (fig. 9d) for sulphamethoxazole respectively. In the light of the previous results obtained with case 6, it was decided to rerun this sample with a gradient of MPCB from 0% to 50% over a period of 30 minutes (effectively increasing the acetonitrile content in the mobile phase from 10% to 60% over 30 min.). Fig. 9e is the chromatogram of the rerun with the new gradient. Paracetamol now had a retention time of 4.85 min. with a 100% match and salicyluric acid gave a retention time of 9.85 min. with a 99.8% match. The absorbance curve of the peak at 10.72 gave a 98% match for sulphamethoxazole. This peak corresponds to the peak at 3.11 min. of the previous chromatogram (fig. 9). Because of the similarity to the spectrum of sulphamethoxazole, these data were tentatively assigned to a metabolite of sulphamethoxazole.

In addition, the peak with retention time of 3.69 minutes was now resolved into two peaks, with similar retention times as were observed in case 6. The UV-spectrum of the peak at 12.39 min. gave a 99.7% match for sulphamethoxazole (fig. 9f) while the peak at 14.55 min. gave a 72.9% match for sulphamethoxazole (fig. 9g) corroborating the findings with sulphamethoxazole in case 6. A paracetamol plasma level of 41.7µg/ml was found on the TDX which confirmed the presence of the paracetamol.

FIG 9

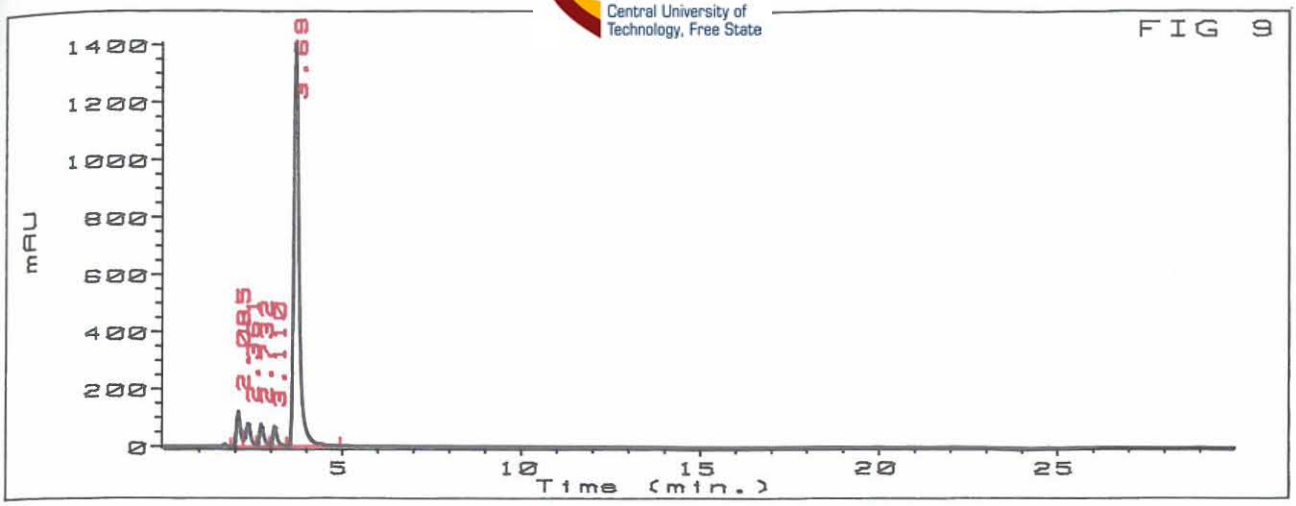


FIG 9a

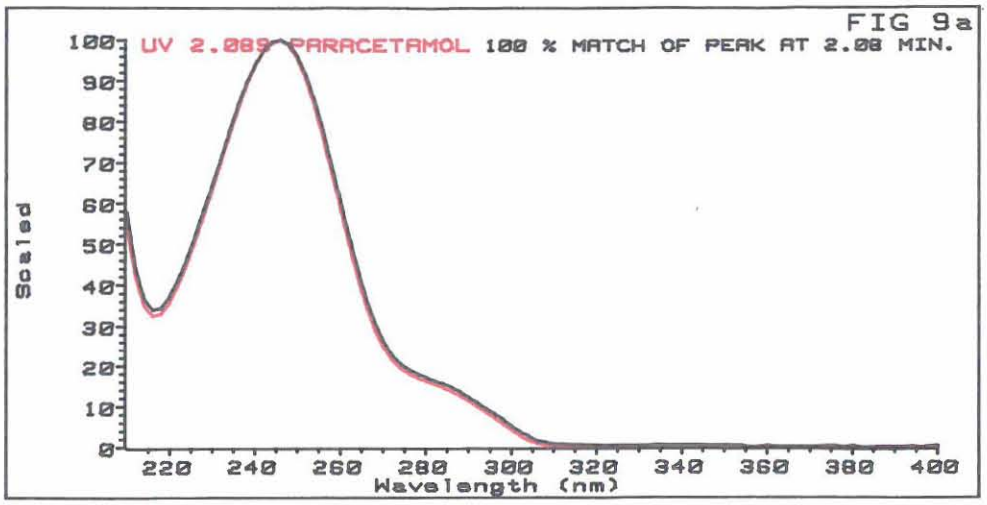
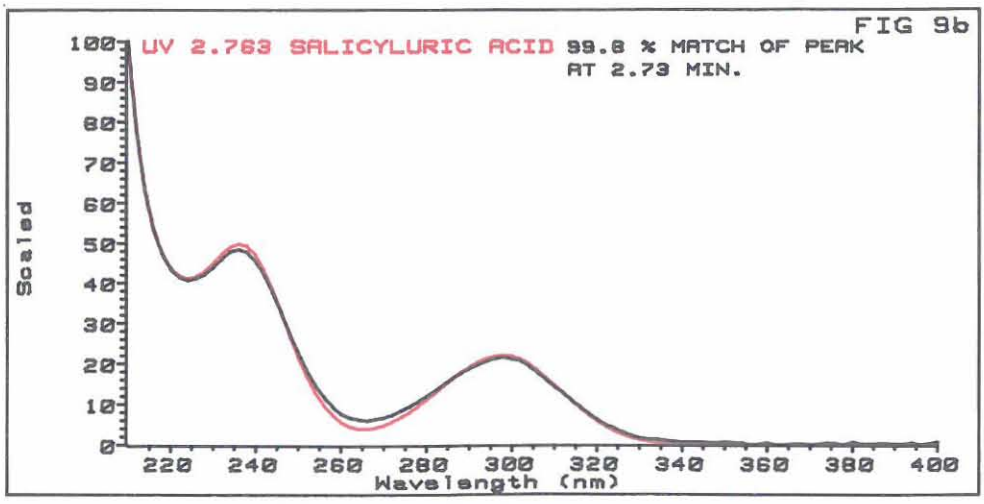
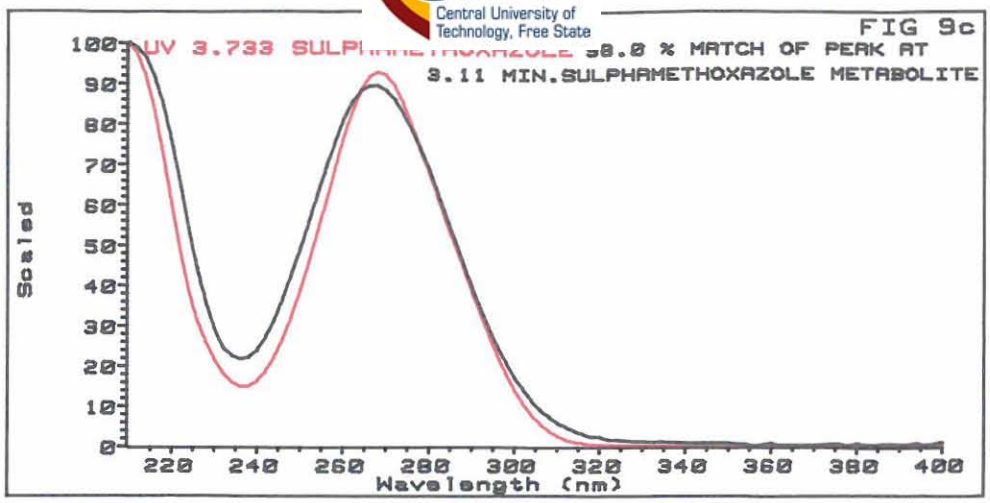
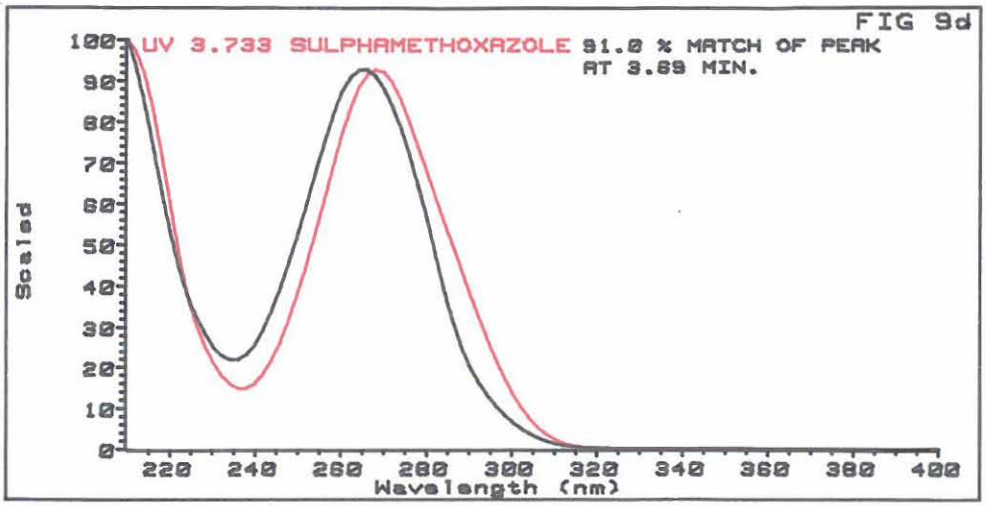
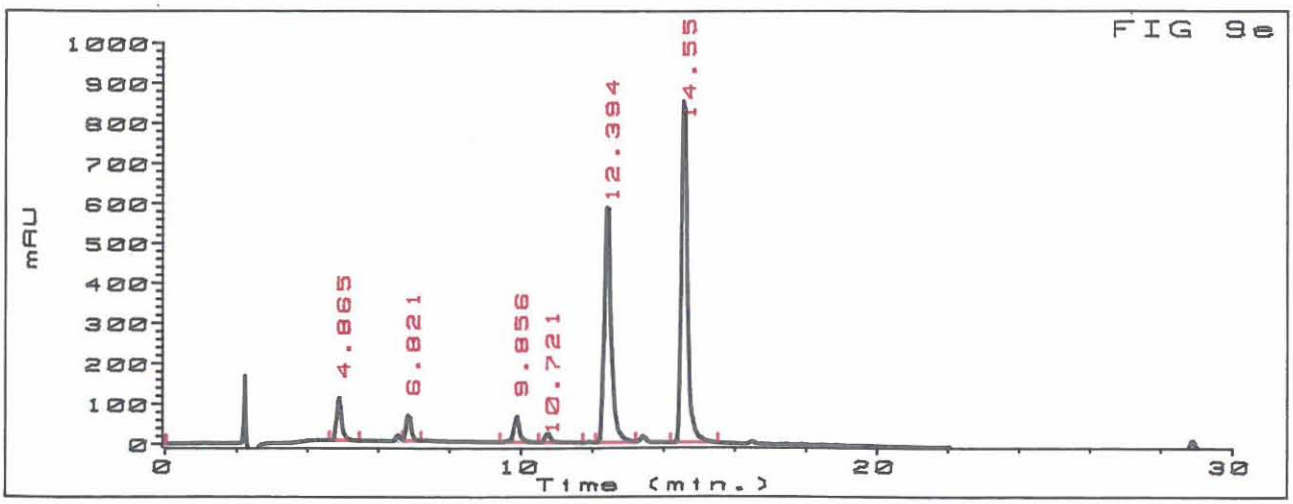


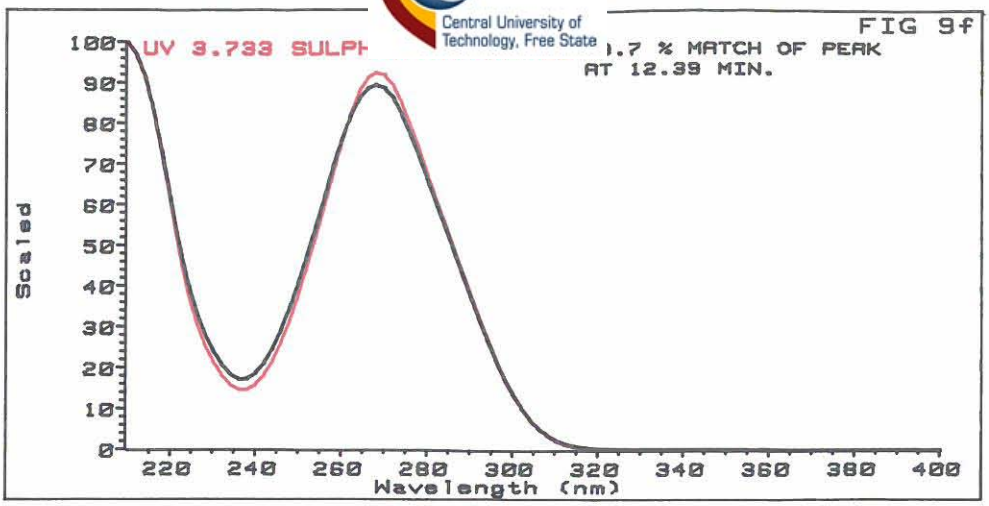
FIG 9b

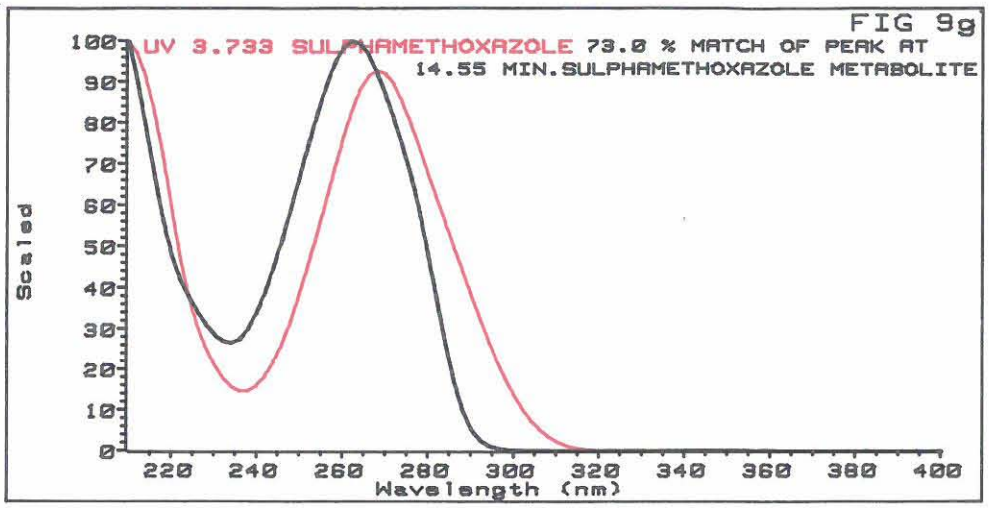












Case 10

Twenty Anadin® (acetylsalicylic acid) tablets were taken by a 26 year old male patient approximately 6 hours before urine and blood samples were collected for analyses. Fig. 10, displaying 3 major peaks, represents the chromatogram which was obtained with the urine extract assayed by HPLC. Fig. 10a is the UV-spectrum of the peak at 2.23 min. This spectrum correlated well with a published spectrum of gentisic acid, a metabolite of acetylsalicylic acid. The spectral data were therefore stored under gentisic acid in the spectral library data base pending corroboration at some later stage. Fig. 10b is the spectrum for the peak at 2.73 min. giving a 100% match for salicyluric acid. A 98.8% match was found for salicylic acid (fig. 10c). Here, once again, the absorbance maxima did not correlate as well as was observed for other compounds. Analysis of a plasma sample of this patient on the TDX gave a salicylic acid plasma level of 450µg/ml, indicating a moderate poisoning with salicylic acid

FIG 10

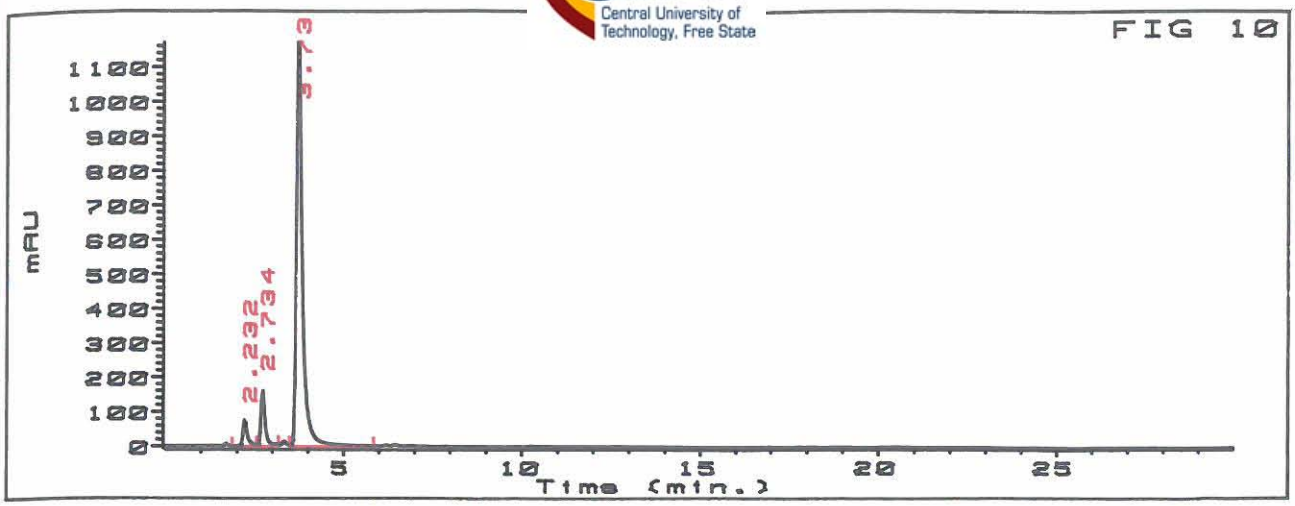


FIG 10a

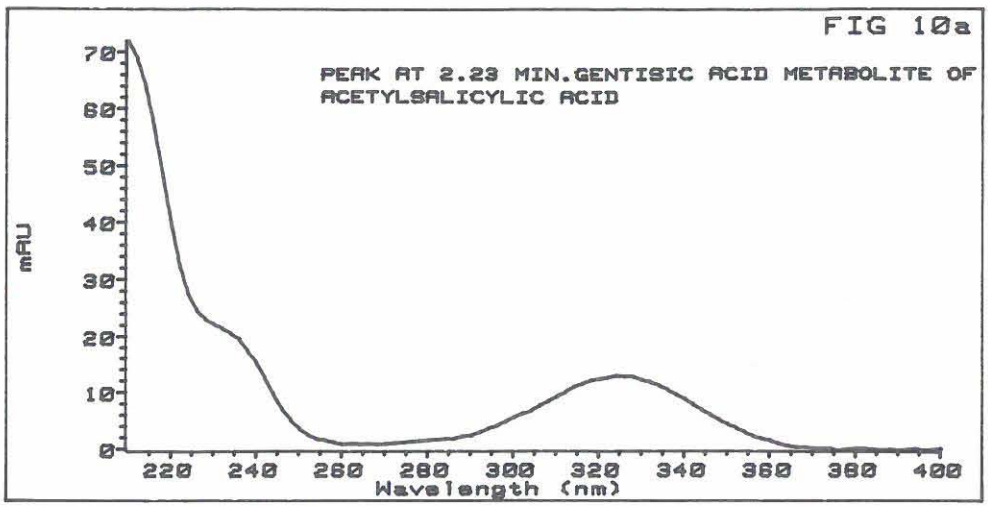


FIG 10b

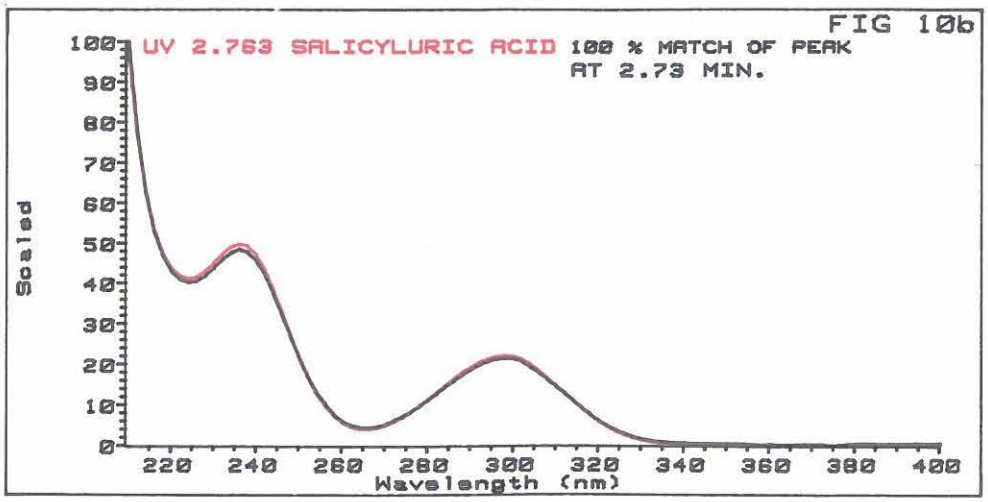
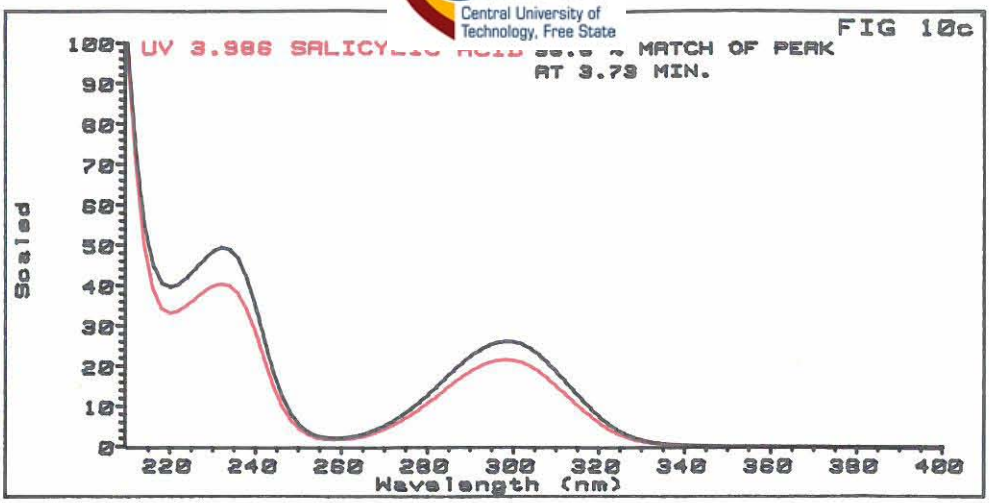


FIG 10c



Case 11

A 27 year old male patient took a suspected overdose of paracetamol. The chromatogram obtained by HPLC of a urine extract (fig. 11) displayed one peak with a retention time of 2.06 min. The absorbance spectrum of this peak gave a 100% match for paracetamol (fig. 11a). The plasma paracetamol level was 51.0µg/ml, as analysed on the TDX.

FIG 11

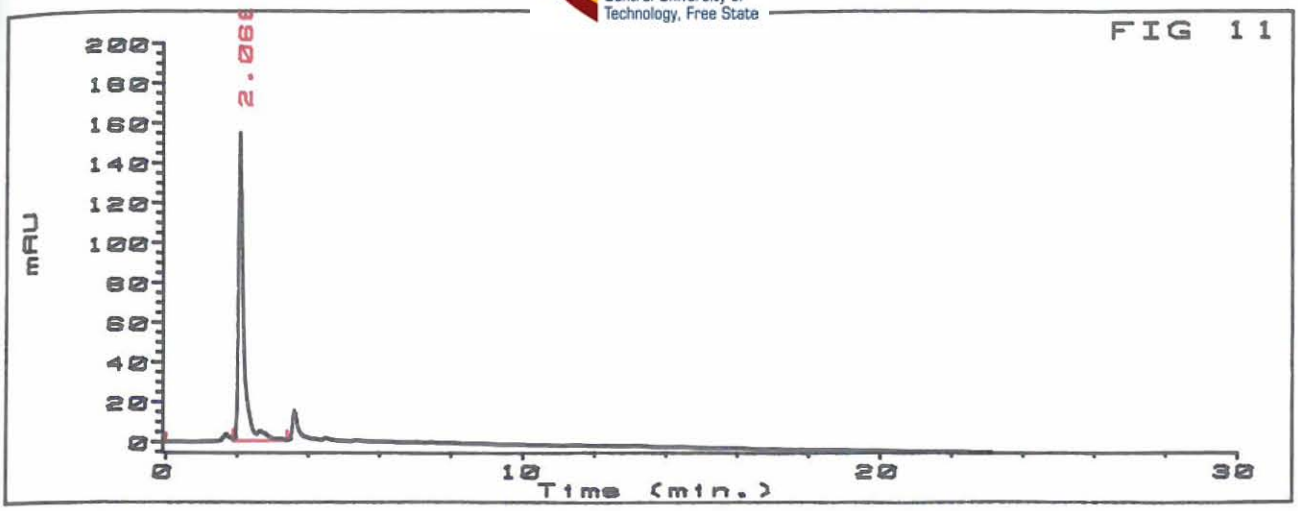
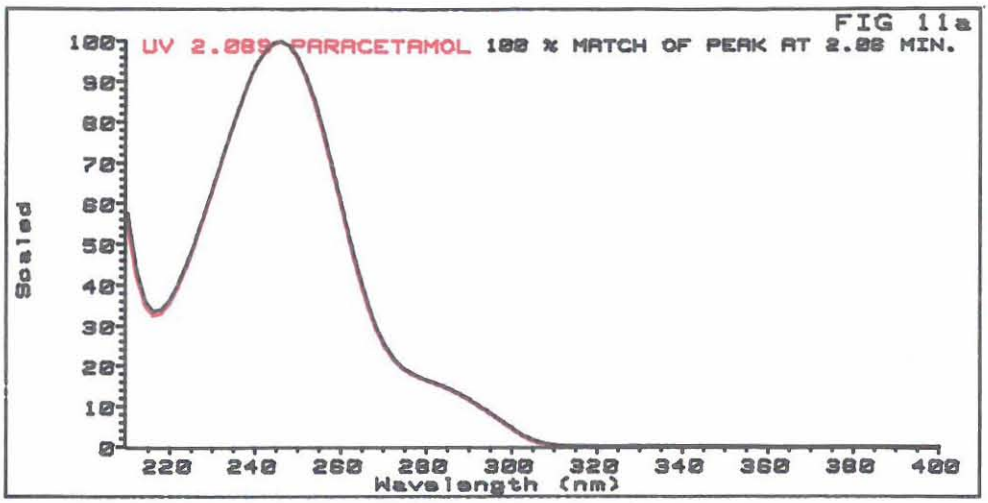


FIG 11a



Case 12

A 38 year old female patient was seen at the casualty department with the history that she had taken various tablets in an overdose. In a gas chromatographic screening for basic drugs, cyclizine, pseudo-ephedrine and nicotine were identified.

Fig. 12 represents the chromatogram of the urine screening for acid and neutral drugs run on the HPLC.: Salicyluric acid (2.74 min.; 99.8% match; fig. 12a), salicylic acid (3.76 min.; 99.8% match; fig. 12b), and ibuprofen metabolite (4.08; 100% match; fig. 12c) were identified with the library search. The spectrum of the ibuprofen metabolite correlated well with previous spectra and retention times of a metabolite of ibuprofen found in cases 2 and 13. This case therefore represents the identification of the ingestion of a specific drug from its metabolite data and not that of the parent substance. An indication of the presence of the parent substance could be observed in the chromatogram (small peak at about 16 min.) but the peak was too weak for a satisfactory UV-spectrum to be obtained for identification by spectral library search.

FIG 12

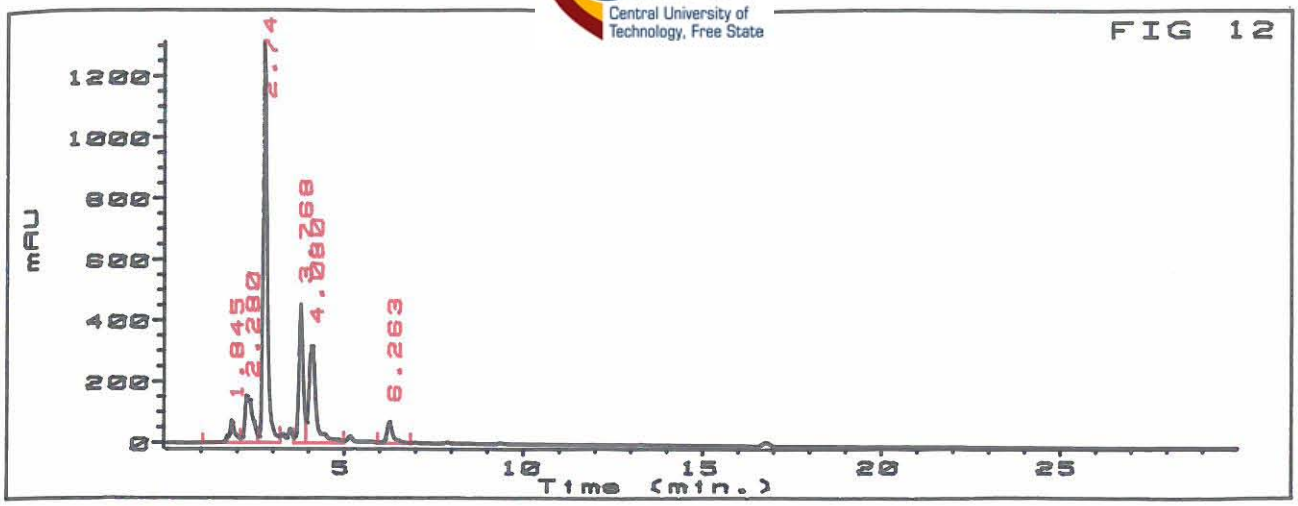


FIG 12a

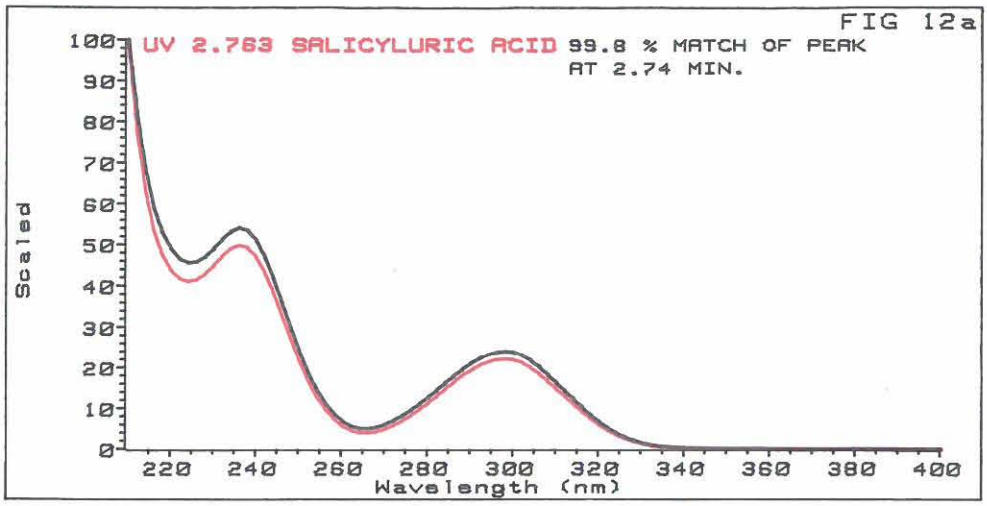


FIG 12b

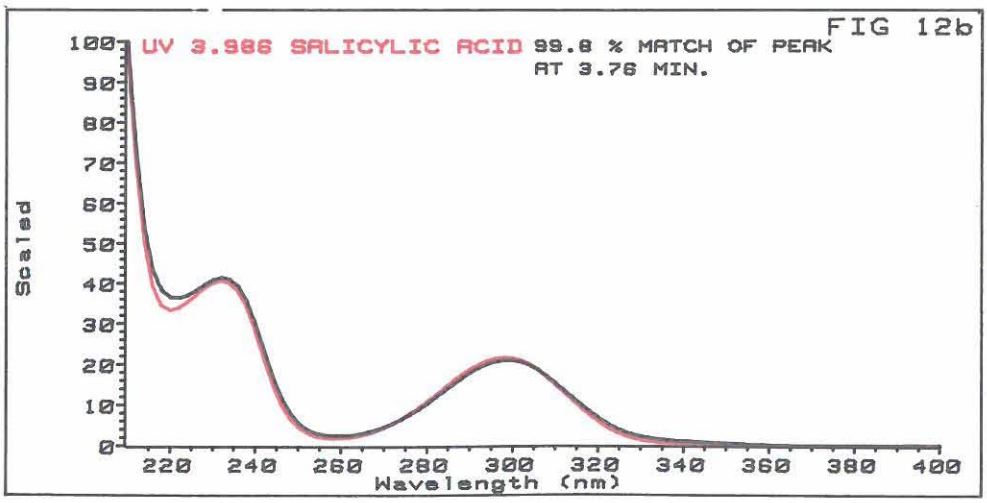
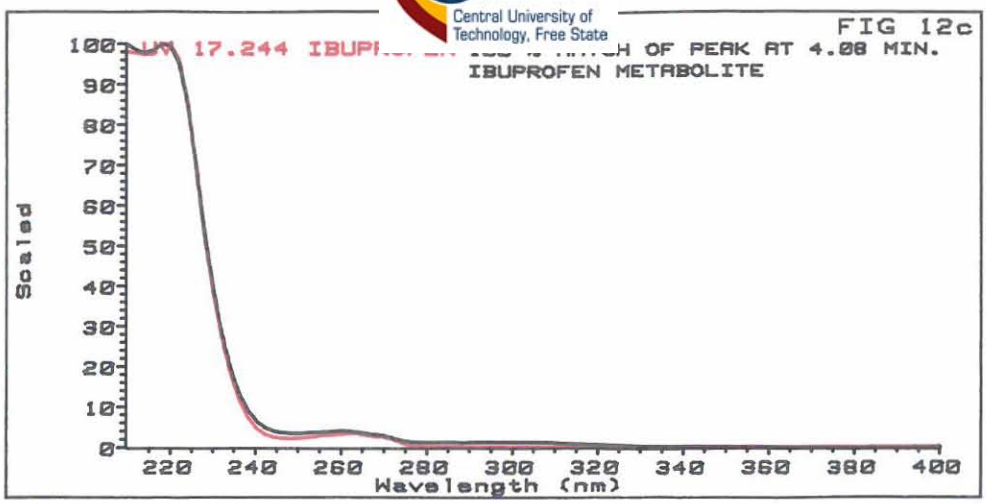


FIG 12c



Case 13

Ibuprofen, (16.83 min.; 99.8% match; fig. 13d), was found in a urine sample sent in for a barbiturate screening. The chromatogram (fig. 13) also displayed peaks at 4.05, 4.46 and 5.16 min. These peaks gave relative retention times with respect to ibuprofen of 0.241, 0.265 and 0.307. These relative retention times correlated well with the data discussed in case report 2. The matches found with the library search are presented in fig. 13a-c. The noise on the UV-spectra is due to the low concentration of these substances and probably indicates that a therapeutic dose of ibuprofen was involved here and not an overdose as was taken in case 2.

FIG 13

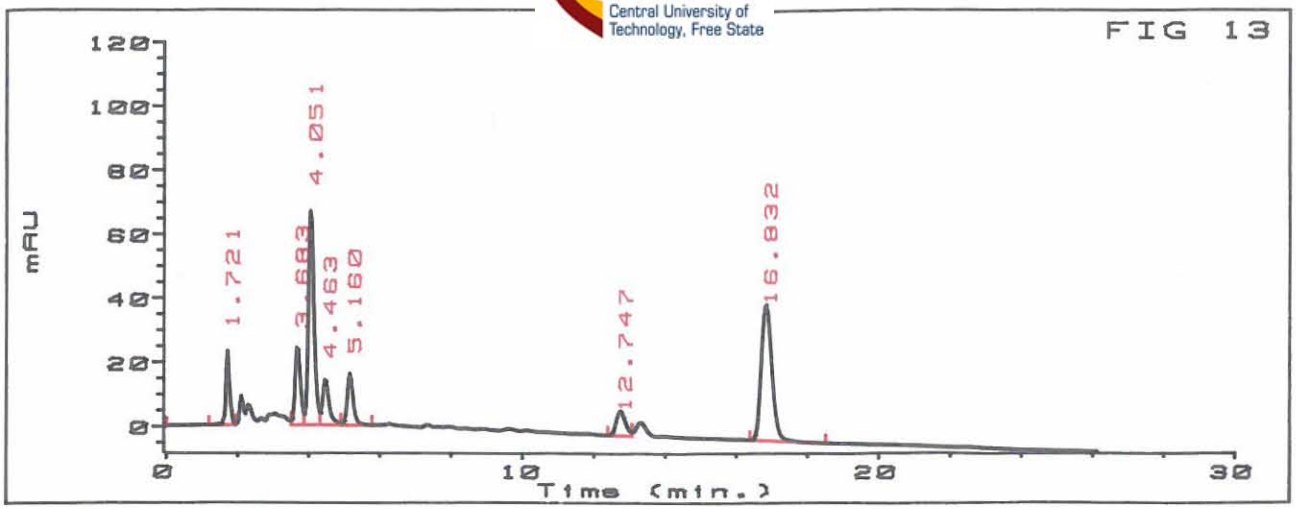


FIG 13a

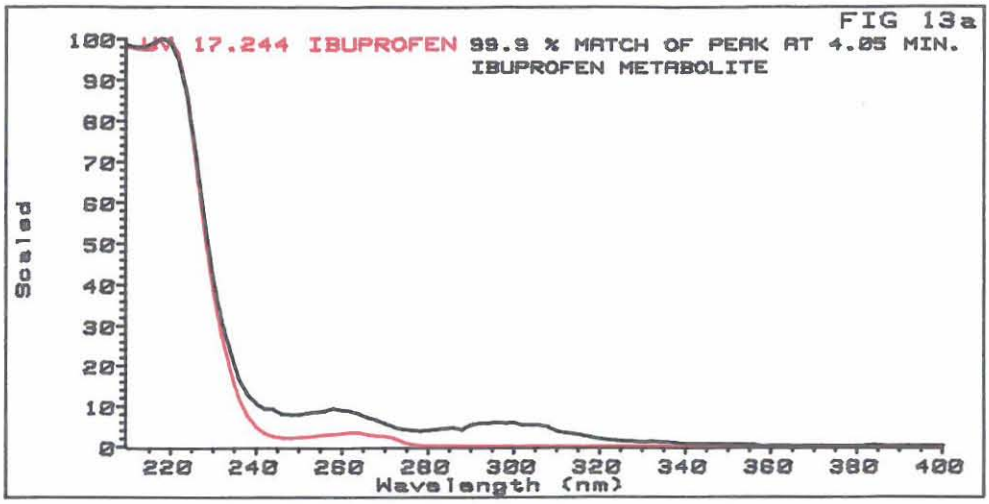
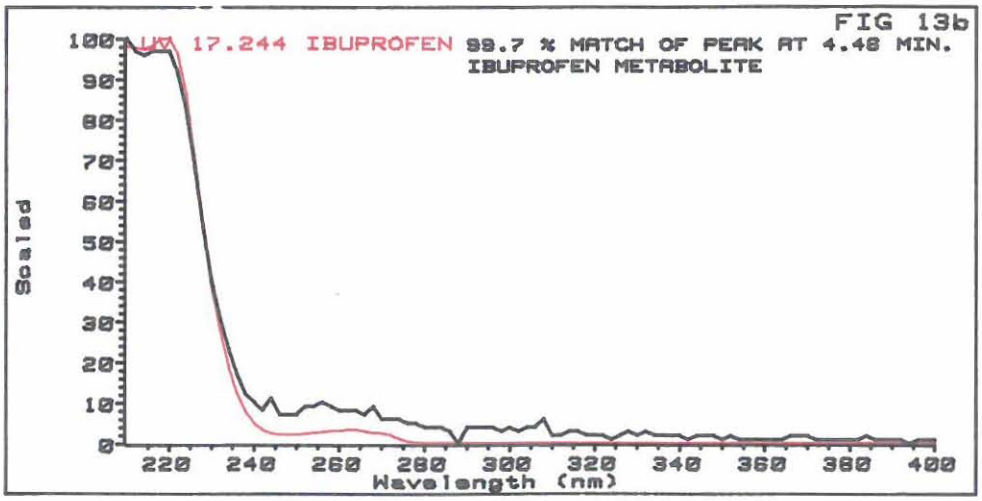
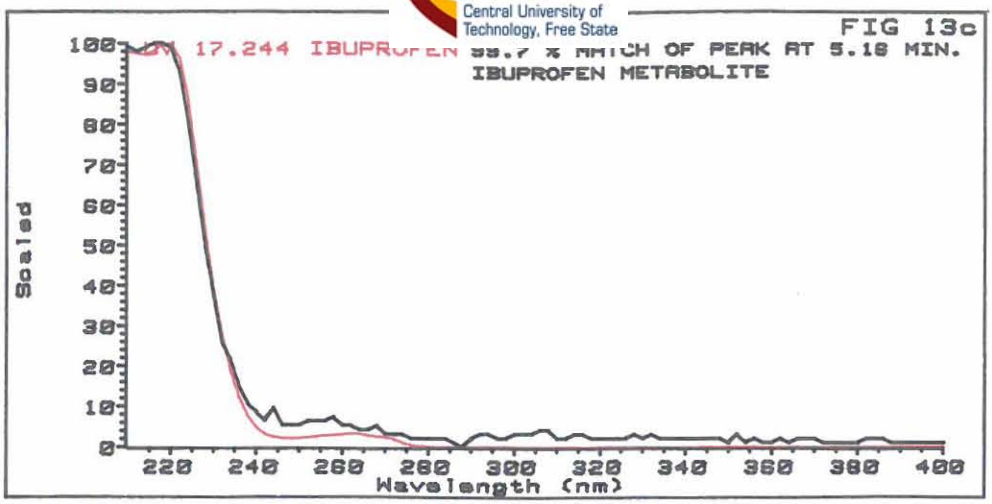
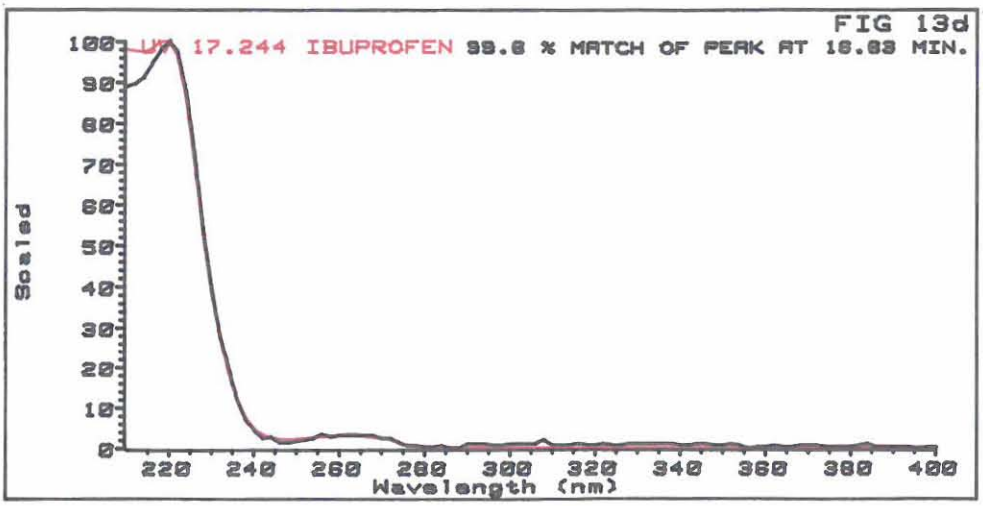


FIG 13b







Case 14

A urine sample was sent in with the request to screen for the presence of diuretics. The sample was collected from a female patient who was suspected of abusing diuretics. Fig. 14 represents the chromatogram obtained by means of HPLC-screening. Only one peak with a retention time of 2.44 min. gave a satisfactory UV-spectrum. The 99.9% match found for hydrochlorothiazide (fig. 14a) confirmed the clinical diagnosis.

FIG 14

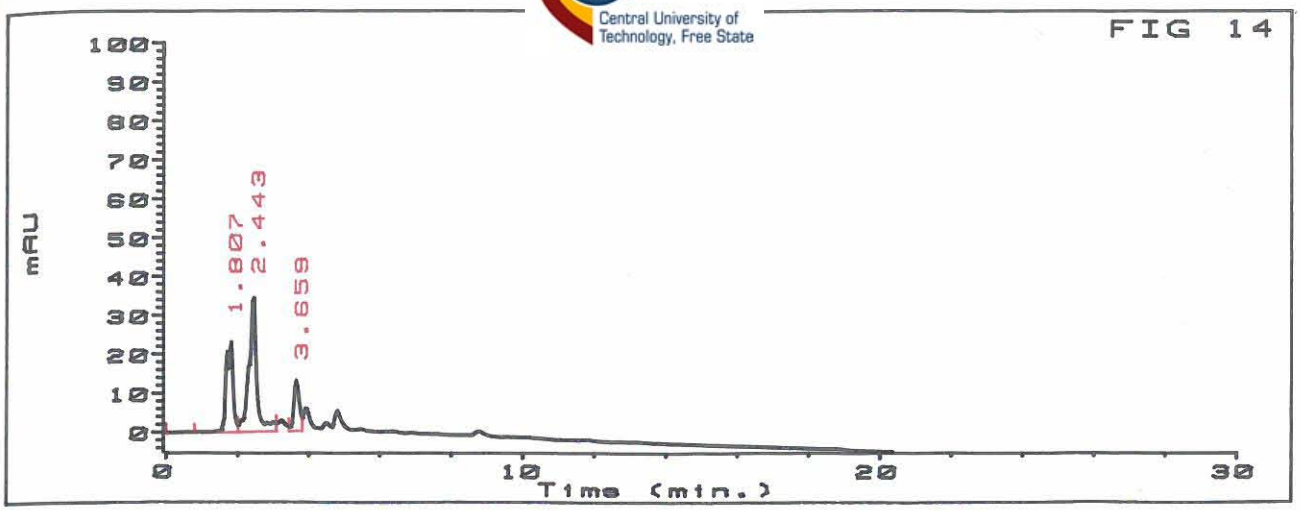
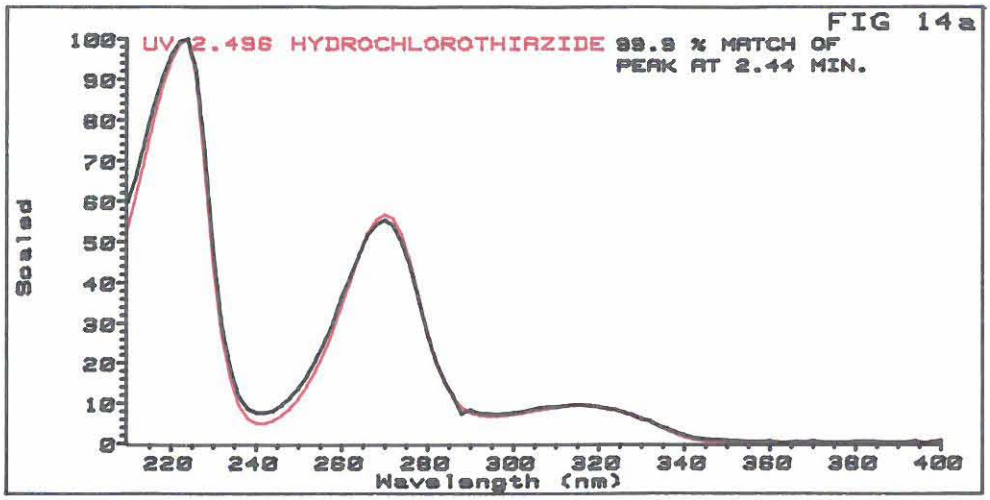


FIG 14a



Case 15

Fig. 15 represents the chromatogram of an extract of urine from a patient taking therapeutic doses of ketoprofen. Fig. 15a shows the match found for ketoprofen (99.9%) for the peak with a retention time of 9.39 min., which indicates that the method will have no difficulties in picking up ketoprofen in an overdose situation. The peak at 6.00 min. gave no satisfactory UV-spectrum and could not be identified.

FIG 15

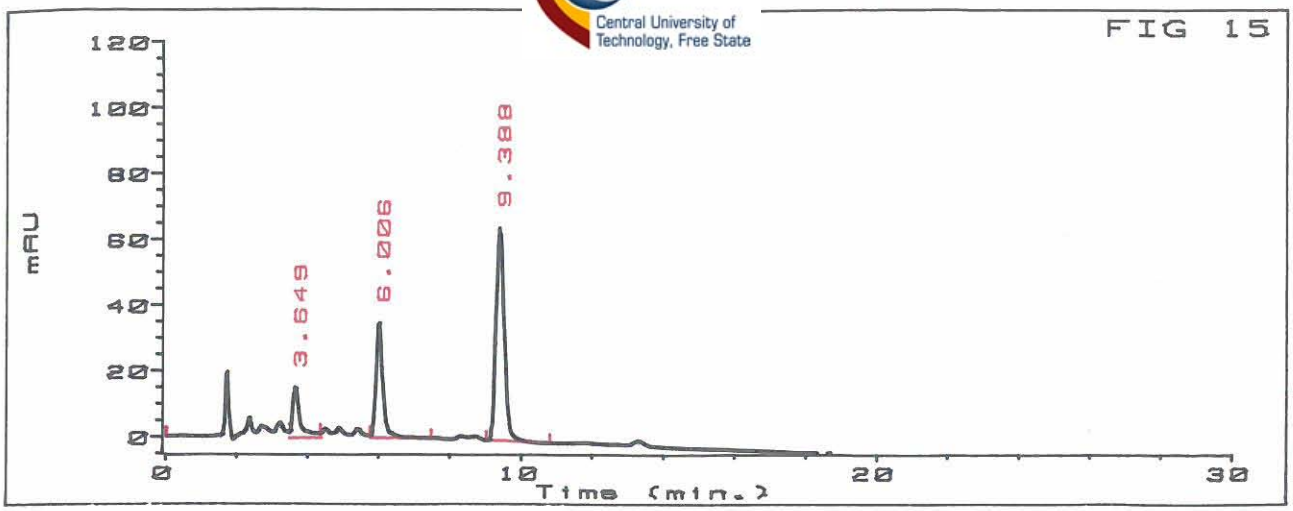
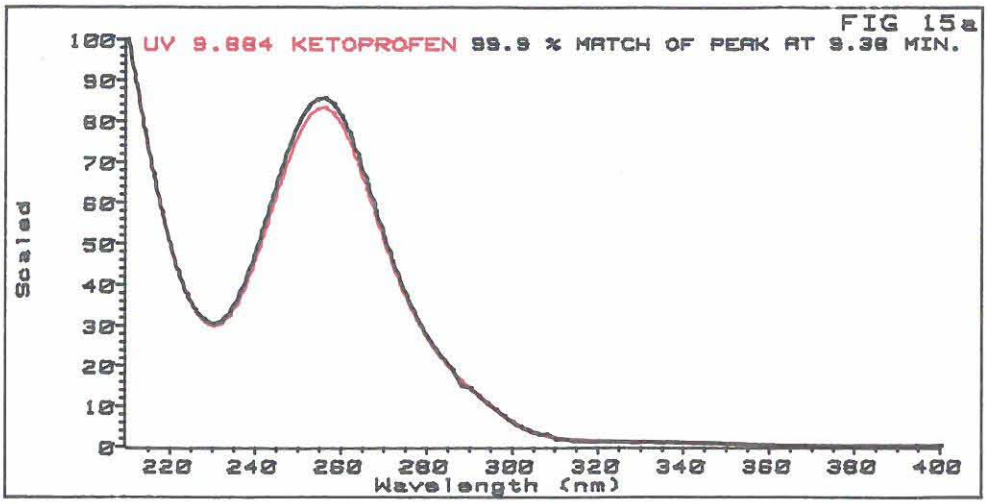


FIG 15a



Case 16

A medication sample was sent to the laboratory for analysis by a local clinician. This medication was dispensed by another clinician for treatment of an overweight patient. The patient had severe side-effects and this laboratory was consulted to find out what the active ingredients of the medication were.

On gas chromatographic screening for basic drugs, norephedrine and caffeine were identified. Fig. 16 is the chromatogram obtained from the HPLC-screening of a methanol solution of the medication.. Four major peaks were observed which were identified as hydrochlorothiazide (2.48 min.; 99.9% match; fig. 16a), caffeine (2.70 min.; 99.8% match; fig. 16b), benzoic acid (3.81 min.; 100% match; fig. 16c), and phenolphthalein (6.65 min.; 100% match; fig. 16d).

FIG 16

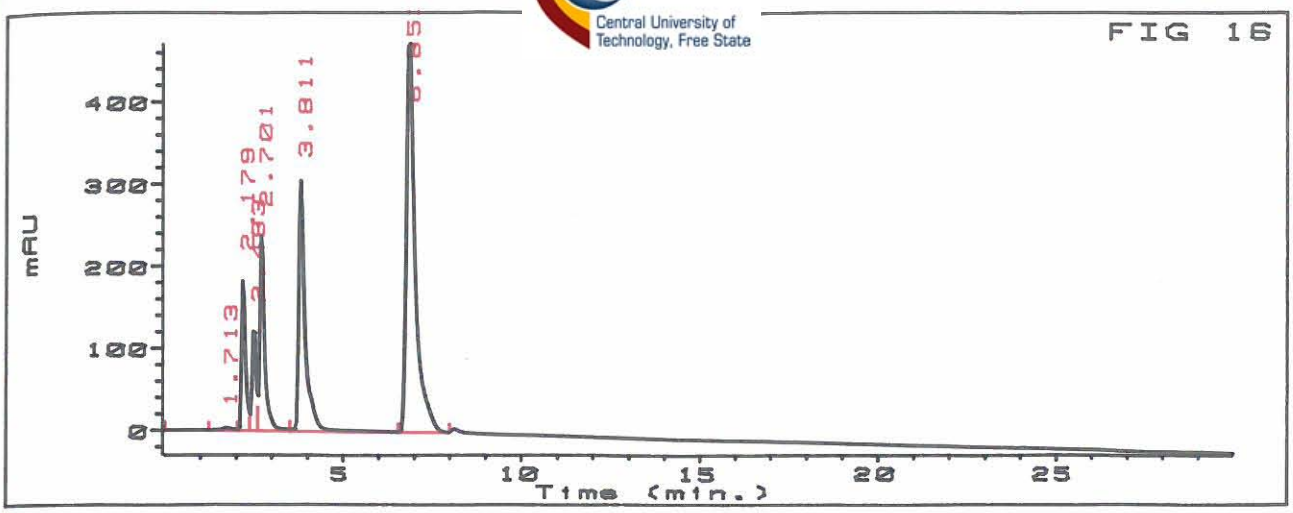


FIG 16a

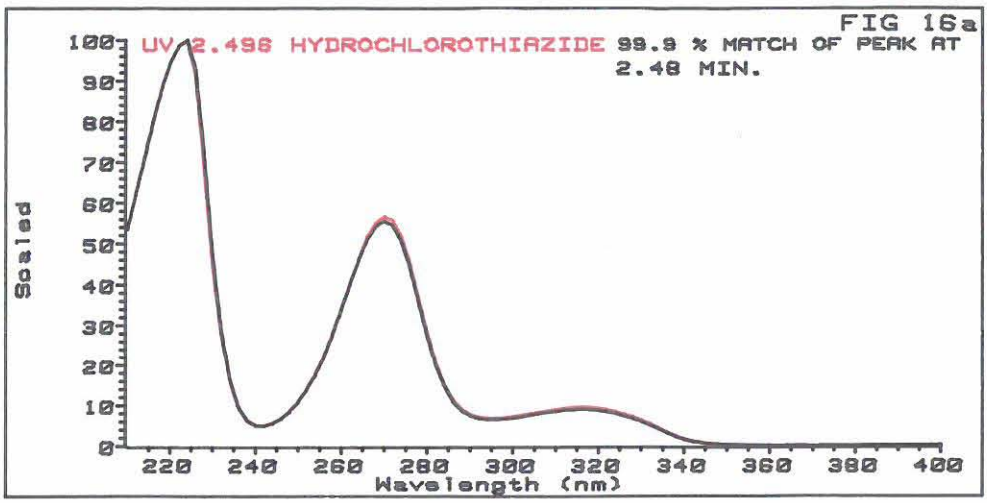
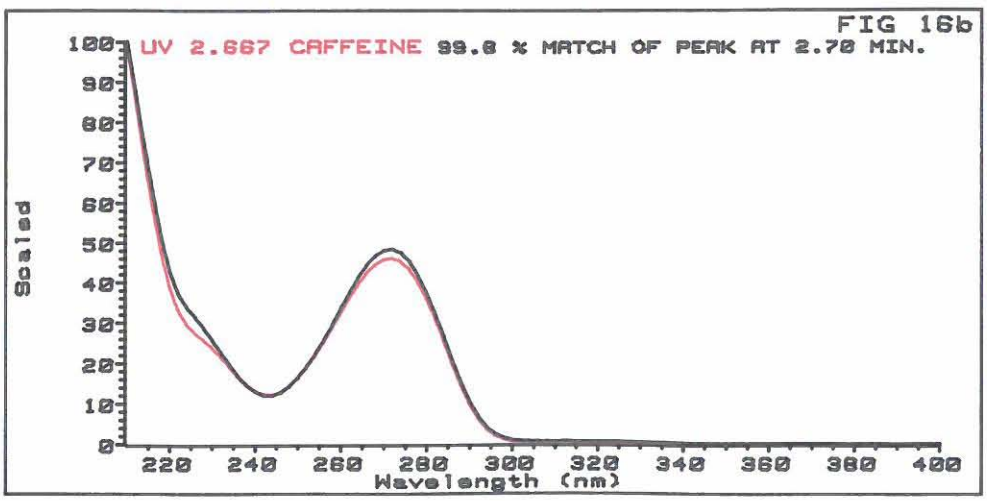
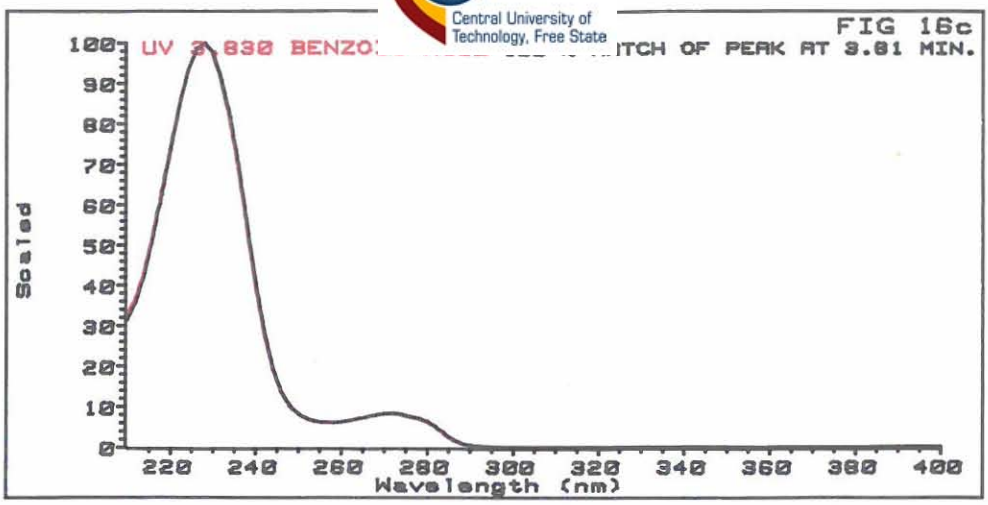
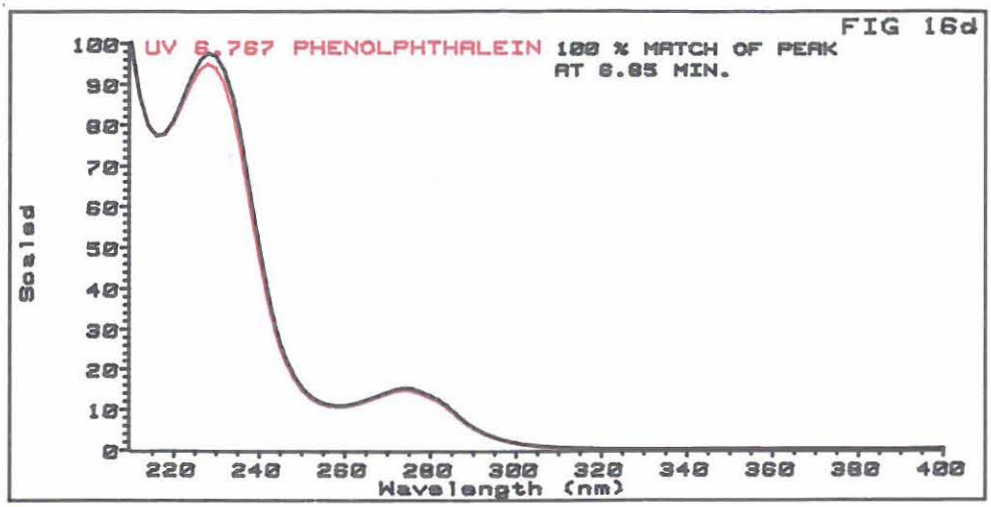


FIG 16b







Case 17

A 17 year old female was admitted to hospital with the history that she had taken an overdose of her grandfather's medication. Her grandfather is an epileptic and the laboratory was asked to screen for anti-convulsants. Blood and stomach contents were sent to the laboratory for analysis. Since a urine sample was not obtained, 1 ml of the stomach contents was used in the HPLC-screening. The same procedure as for the urine extraction, was followed. Fig. 17 represents the chromatogram obtained with the HPLC-screening performed on the stomach contents. Three major peaks were observed. The peak at 2.01 min. gave a 100% match for theophylline (fig. 17a). The theophylline plasma level, determined after theophylline had been identified in the stomach contents, was 50 μg/ml (TDX) which is in the highly toxic range.

The peak with a retention time of 5.31 min. did not give an UV-spectrum with much detail but, because the possibility of anti-convulsants was indicated, the peak match of 99.9% with a spectrum of amylobarbitone was considered important enough to confirm the presence of amylobarbitone by GC-MS. A plasma amylobarbitone level of 7.0 μg/ml was found, using gas chromatography with nitrogen/phosphorus selective detection. The peak at 6.91 min. gave a 100% match for carbamazepine, another anti-convulsant (fig. 17c). The carbamazepine plasma level was found to be 5.1 μg/ml on the TDX.

This case again illustrates the importance of proper toxicological screening and that clinicians should not only rely on the history that is given by the patient or accompanying persons. Had the clinician's request for target screening for anti-convulsants only, been heeded, carbamazepine and possibly amylobarbitone would have been identified and theophylline would certainly not have been detected. The clinician would probably have been satisfied that the patient's condition was due to an overdose of amylobarbitone with the plasma level of carbamazepine being in the lower therapeutic range. Fortunately the patient was probably protected against the potentially lethal concentration of theophylline

by the presence of the two anti-convulsants, and had the presence of theophylline not been detected, nobody would have been any the wiser.

FIG 17

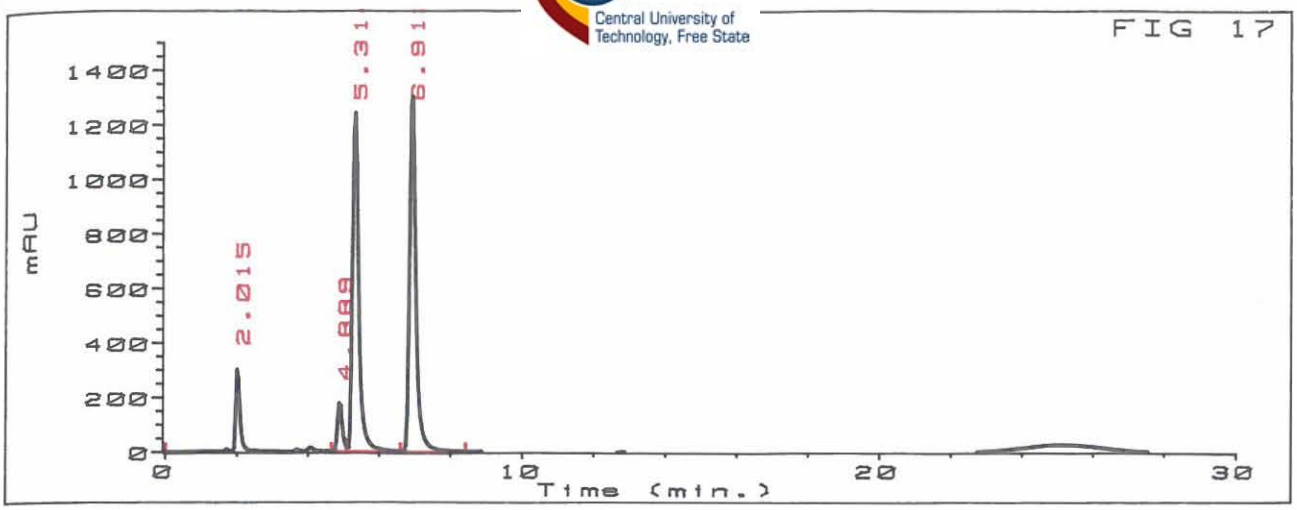


FIG 17a

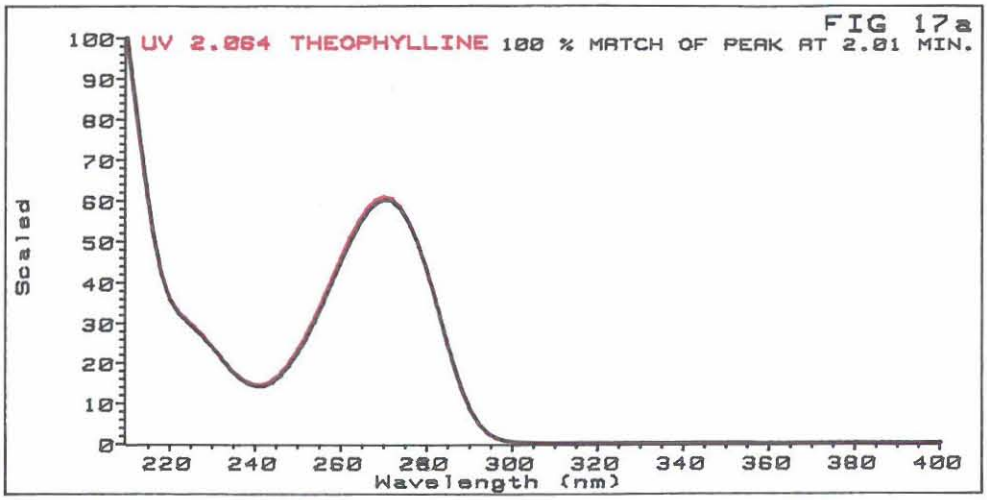


FIG 17b

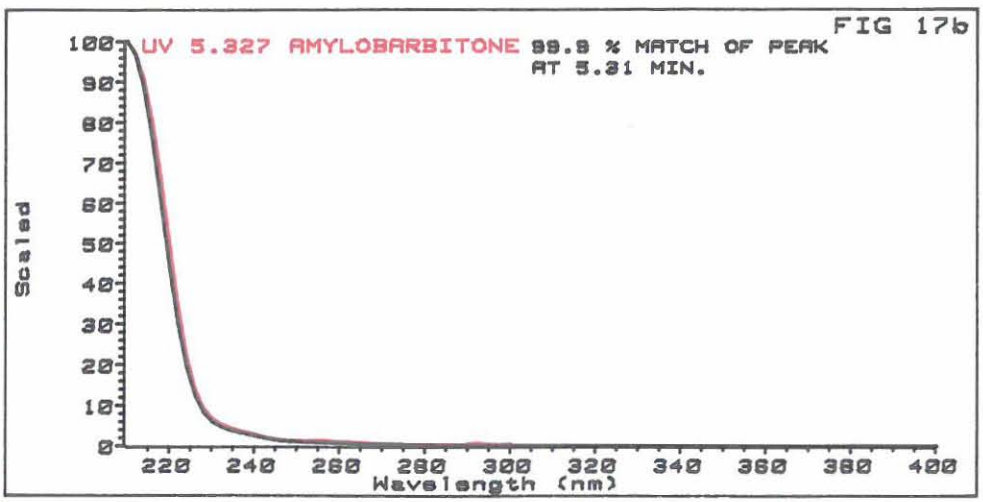
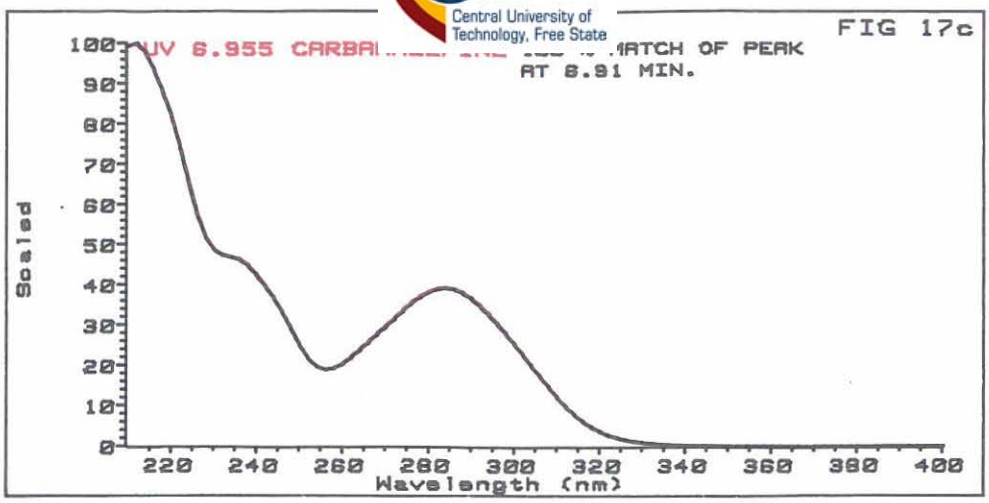


FIG 17c



4.4 General discussion

The liquid-liquid extraction with diethyl ether, as organic solvent, followed by the lead acetate wash resulted in a clean extract with a minimum of endogenous compound interference with the chromatography of the drugs studied. The extraction yields were good for a wide range of compound types, illustrating the universal applicability of the liquid-liquid extraction procedure compared with the solid phase extraction which is often quite specific. This specificity of the solid phase extraction procedure is of course an advantage when quantitative assay procedures are sought for specific drugs but not in the case of a general qualitative screening procedure. The lead acetate wash step did not adversely affect the extraction yield of any of the compounds tested. The cost of materials for the liquid-liquid extraction was a small fraction of what the costs of a solid phase extraction procedure would have amounted to.

The mobile phase gradient elution system turned out to be as versatile as expected, yielding well-shaped peaks for all the drugs tested and even for a number of basic compounds which were identified in the course of some of the case studies. This feature warrants a study to investigate the applicability of this chromatographic system to basic drugs in general.

Sixteen different substances, excluding their metabolites, were identified in the seventeen case reports described. The matches, ranged between 98.8 % and 100 % for the parent substances for which spectra of standards were stored in the spectral library. Matches of metabolites compared with the parent substances ranged between 73.0 % and 100 %. The close resemblance between some of the metabolites and their parent substances was remarkable and was an indication that the differences in molecular structure must have been small or that the information content in some of the UV-spectra was rather limited. However, in combination with the retention times of the components, the identifications were always unambiguous.

Four metabolites of ibuprofen (structures unknown), one of naproxen (probably desmethyl naproxen), one of sulphamethoxazole (structure unknown) and three of acetylsalicylic acid (salicylic acid , salicyluric acid and gentisic acid) were detected and stored as metabolite data in the spectral library for future reference. Whenever such metabolites are detected in future case studies, the information will be stored in the spectral database to aid in future identifications. This kind of invaluable information can only be available for direct identification purposes when the screening procedure and the chromatographic and spectral data are generated in-house for reference purposes , which was the main aim of this project. An interesting observation was that, although carbamazepine was identified in three cases, the two major metabolites namely the dihydroxy metabolite and the epoxide metabolites were not detected. This aspect still requires investigation since there is no reason to believe that these metabolites are not extractable by the procedure used.

Five additional compounds, not described in the case reports , were identified. These were tetracaine, bezafibrate, benzoic acid and caffeine. Tetracaine and benzoic acid were identified in a sample of a drug product handed in for identification. Although it is not the intention to broaden this screening procedure to include basic compounds, the fact that tetracaine, a basic compound, could be chromatographed and identified successfully, attests to the versatility of the chromatographic system for other than acidic and neutral compounds. The identification of bezafibrate was made in a urine sample of a patient who ingested normal therapeutic doses giving some indication of the sensitivity of the method.

Stomach contents as biological sample, treated in the same way as the urine samples, was shown to be a useful medium for the extraction and identification of unknown substances and could be used if no urine is available. The use of stomach contents has the advantage that high concentrations of substances are normally present and sensitivity of the method would not be a problem.

For chromatographic resolution of low retention time compounds, such as the sulphonamides, a mobile phase gradient starting with 10% acetonitrile can be implemented whenever necessary and was in fact used in two cases to separate sulphamethoxazole from one of its co-eluting metabolites (case reports 6 and 9)with good results. The change to a different mobile phase gradient only requires a few keystrokes prior to the injection of the extract.

The application of the developed screening procedure to several case studies has confirmed its utility in not only identifying the series of compounds chosen as a core of standards, but also unknown compounds. In addition, the compounds tentatively identified as metabolites in some of the case studies will serve as very important additional corroborative evidence in similar cases in future. This is especially so with drugs which are metabolised to several major metabolites, producing a very distinctive metabolite pattern which makes the evidence of intake of that particular drug irrefutable.

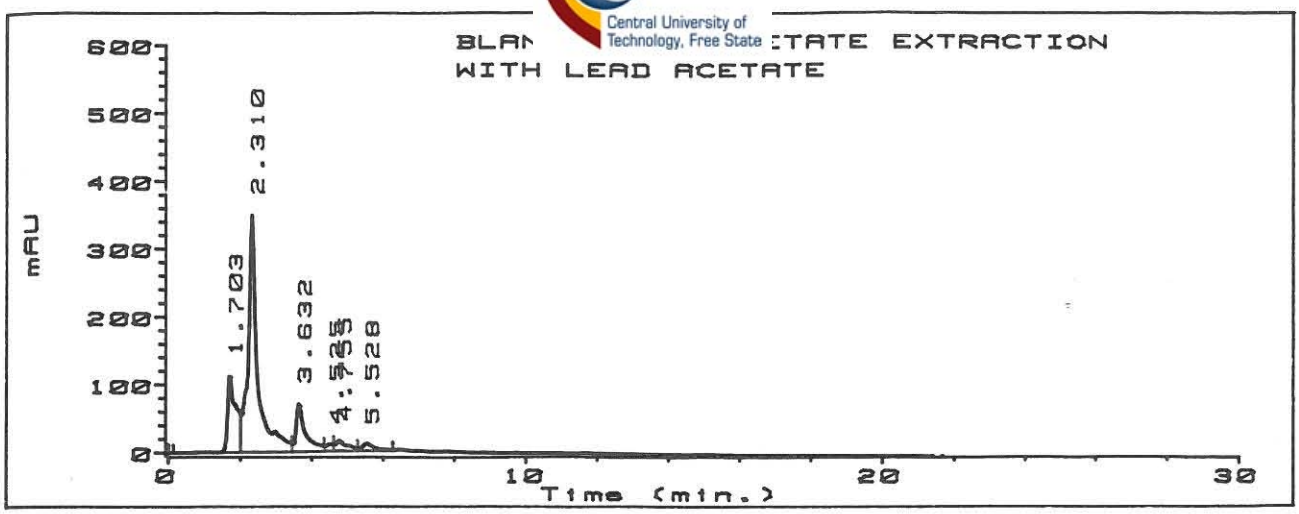
Only in one case investigated so far was a major component in the chromatogram not identifiable. It is possible that this component could have been a metabolite of ketoprofen but this will have to await verification either by way of an administration study or another overdose case involving ketoprofen.

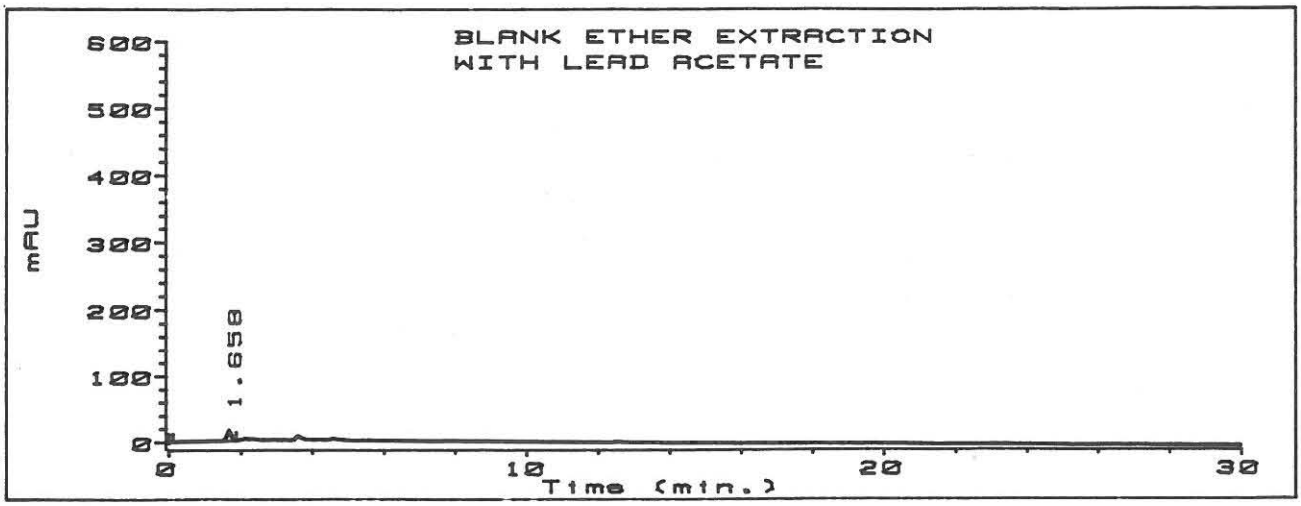
The results obtained with some of the case studies, in which unsuspected drugs were identified, confirmed our contention that all poisoning cases should be studied employing broadly based screening procedures rather than selective testing with commercially available immunoassay based quick tests. Such quick tests are very useful in cases where circumstantial evidence for the intake of a specific drug is very strong but they should never be employed to the exclusion of more general screening procedures. The case in which theophylline was detected in the urine and then quantified in the plasma using one of these quick tests clearly illustrated the proper use of these procedures. If this case had had a fatal outcome with theophylline not being detected there might well have been

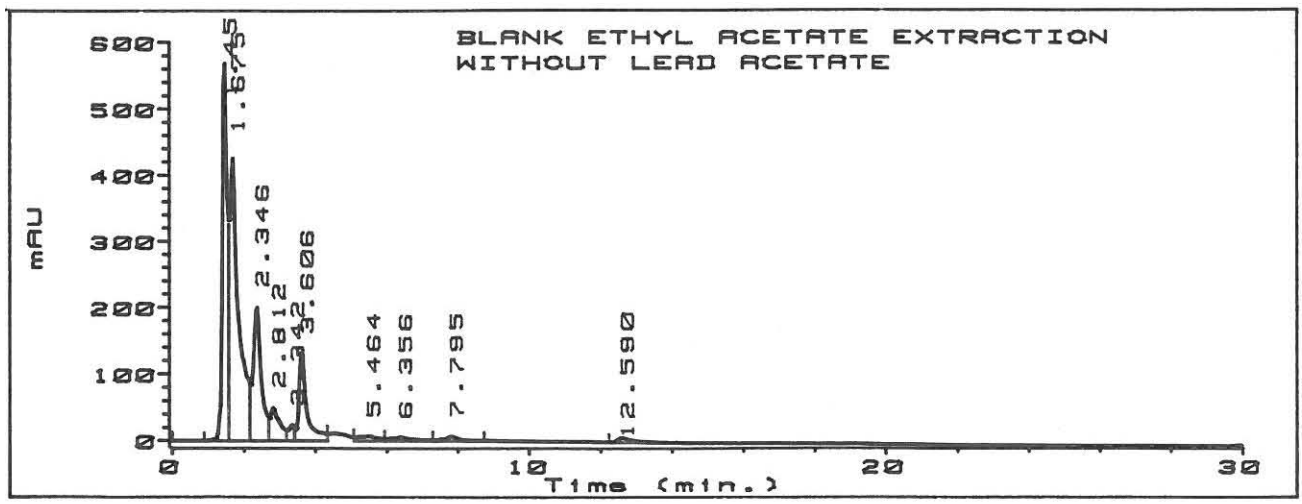
some awkward questions to answer if it were detected during a judicial post-mortem investigation.

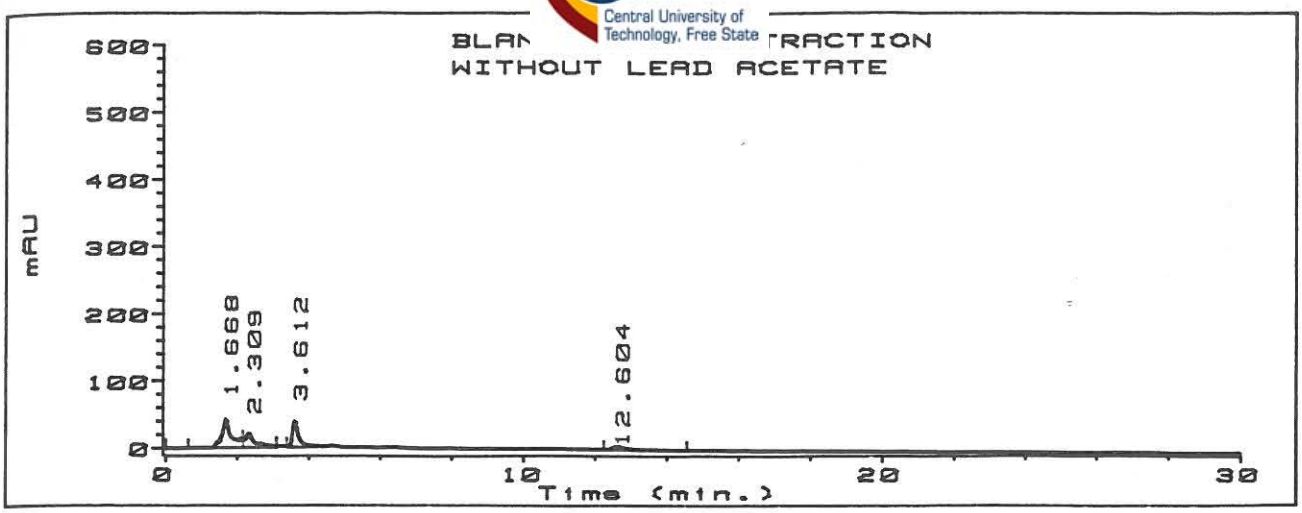
The cumulated information which will be added to the spectral data base while this drug screening procedure is being used in future cases, will eventually constitute the real contribution of this developmental research project.

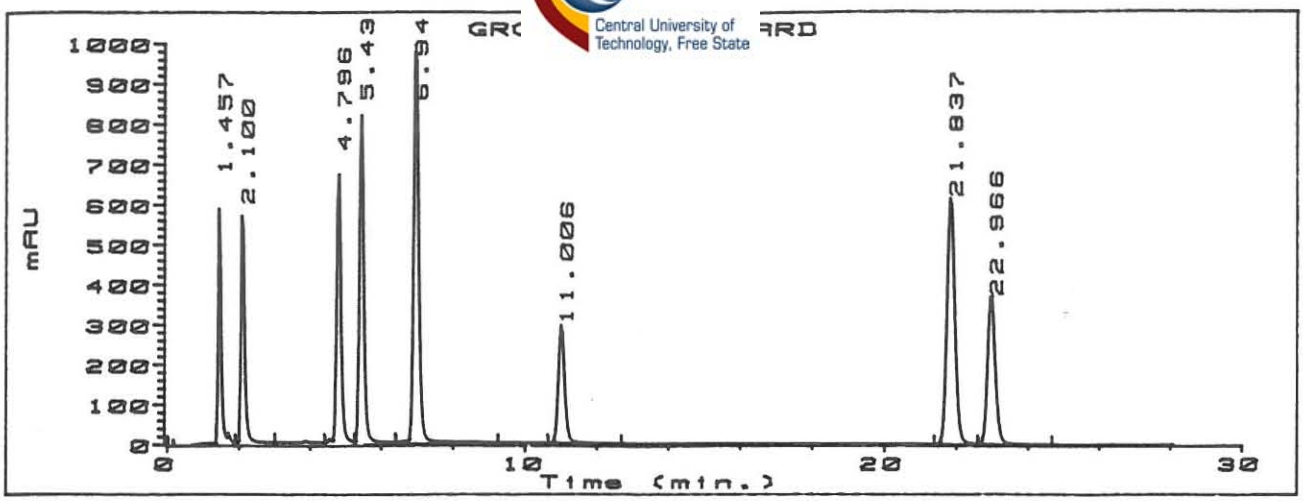
APPENDIX I

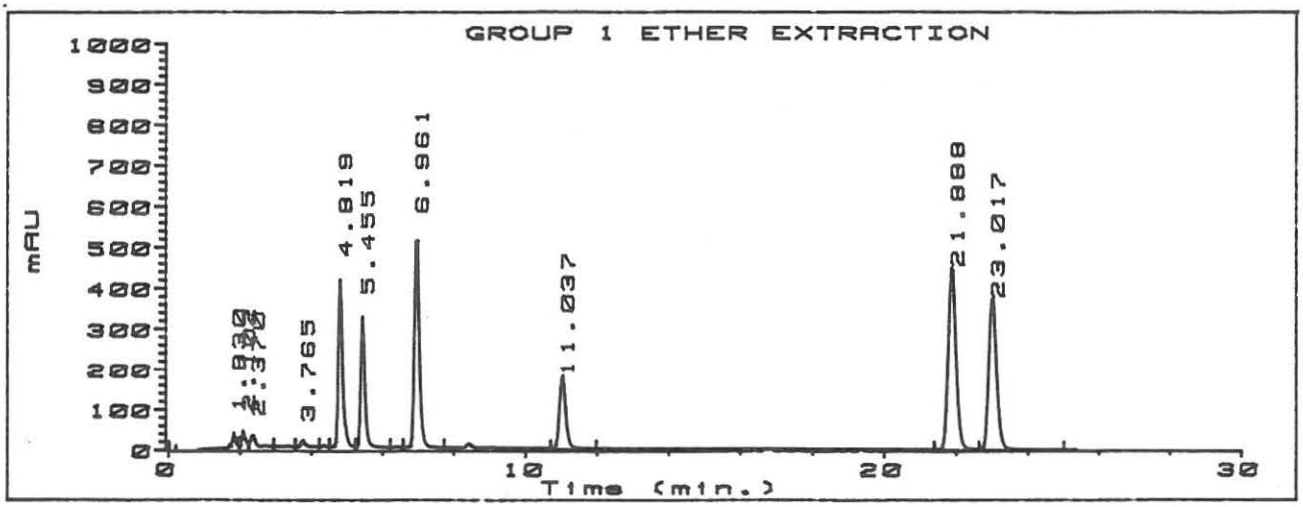


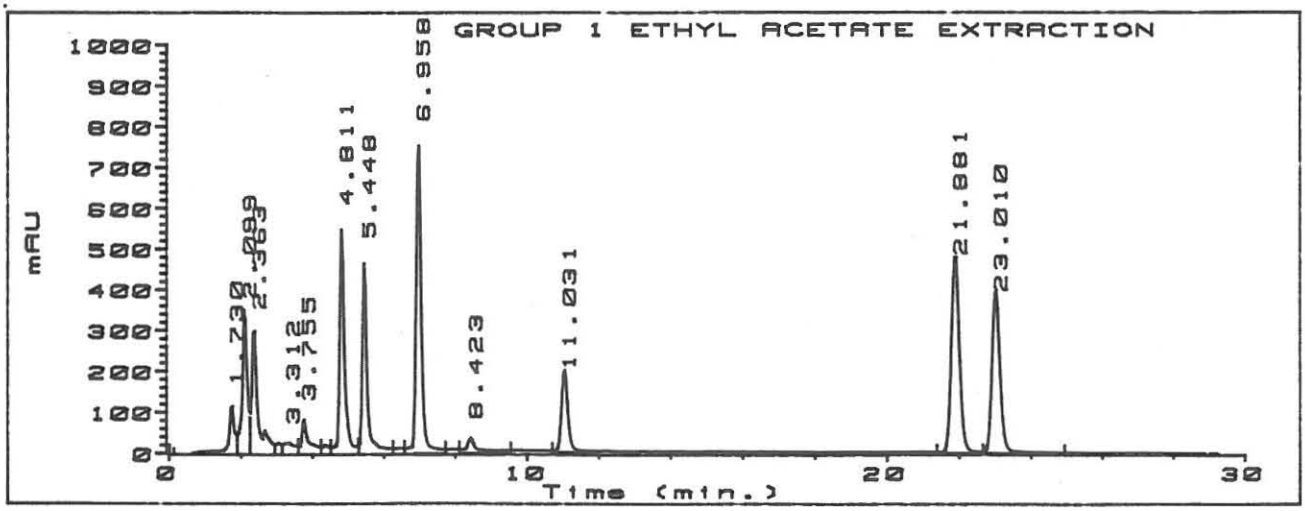


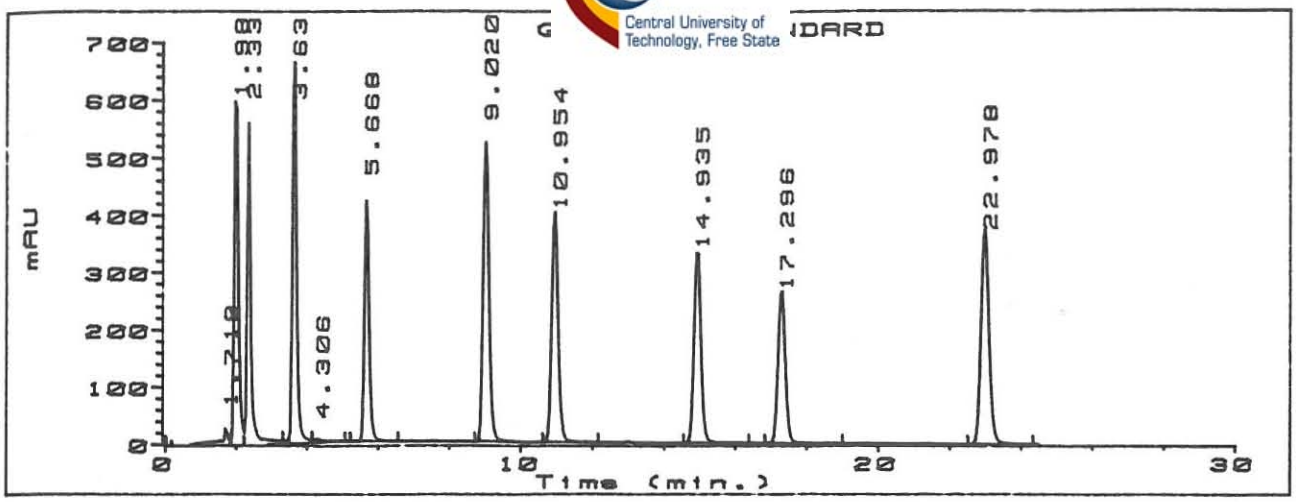


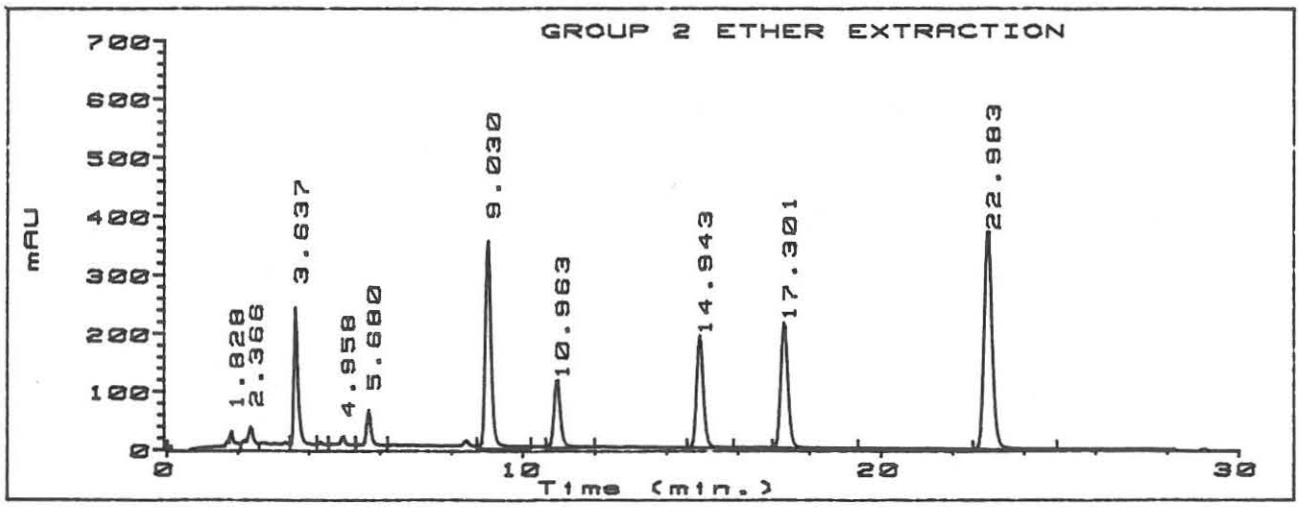


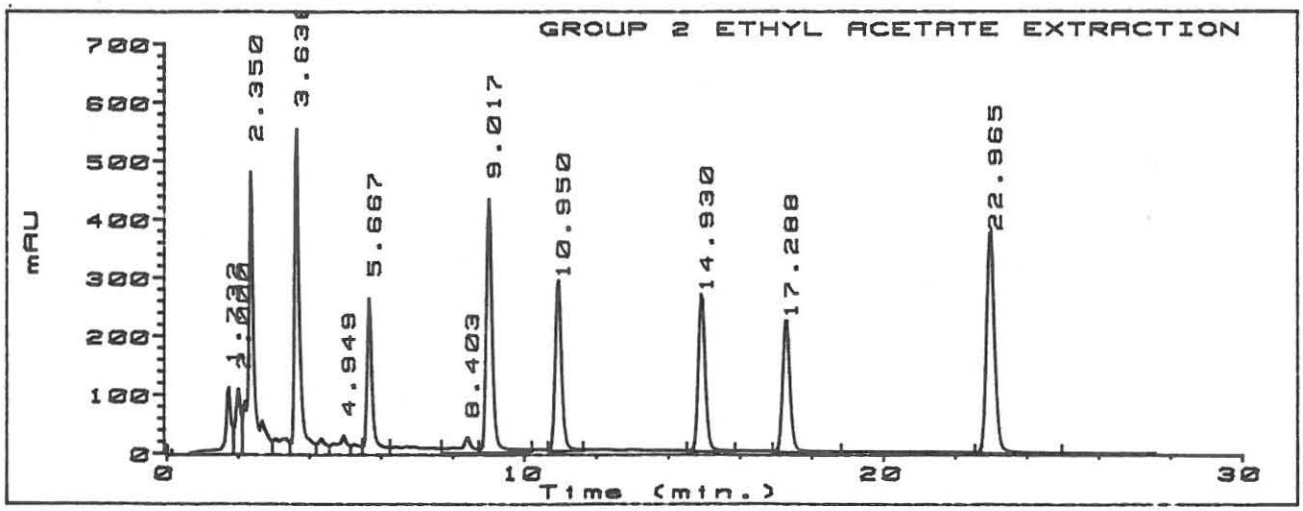


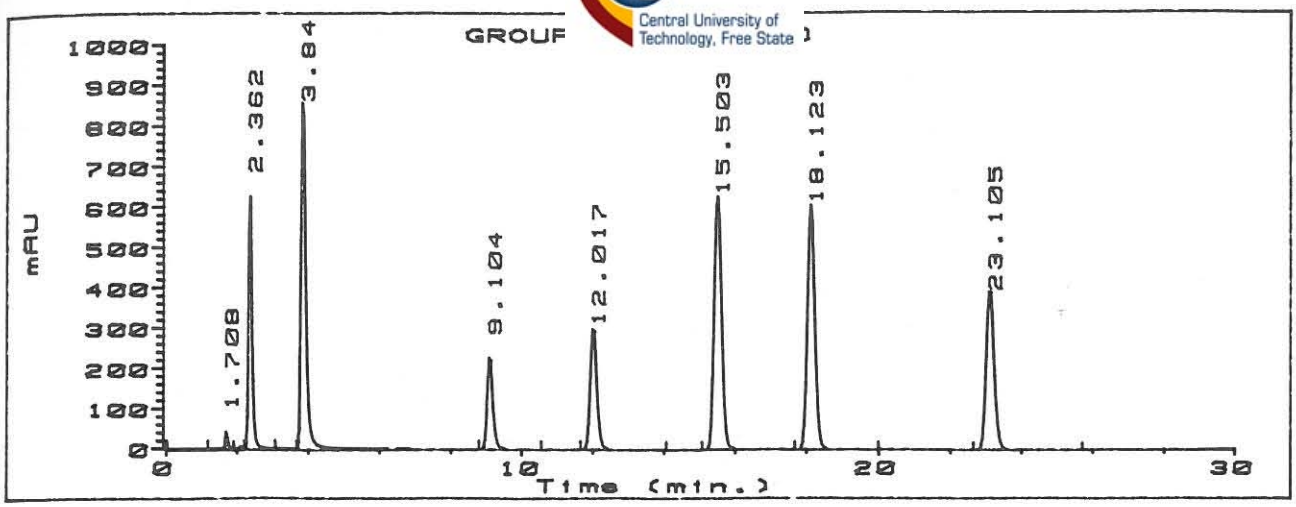


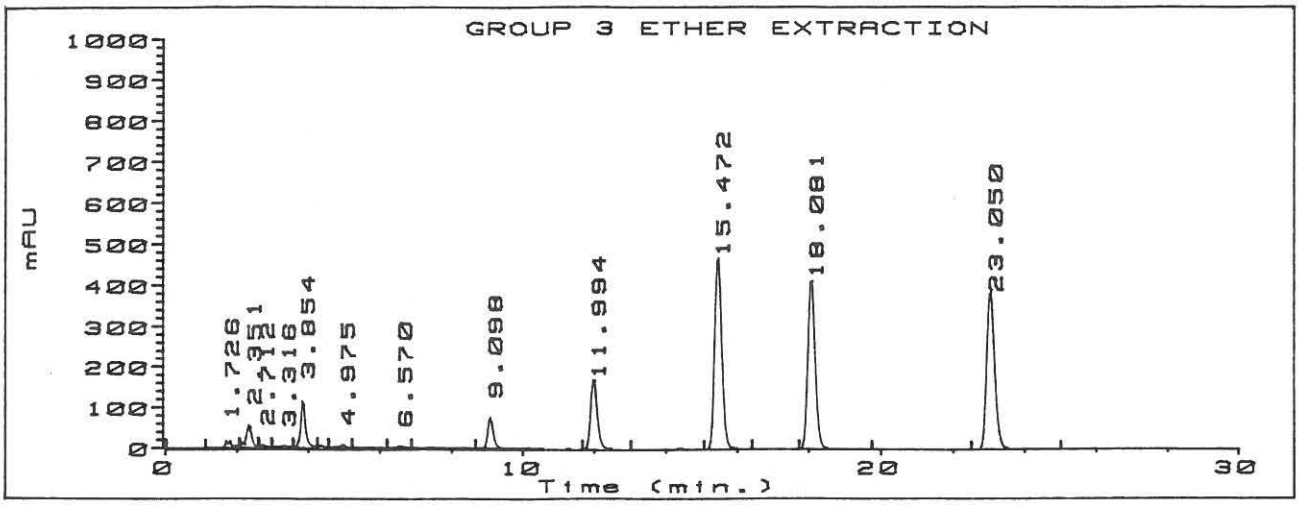


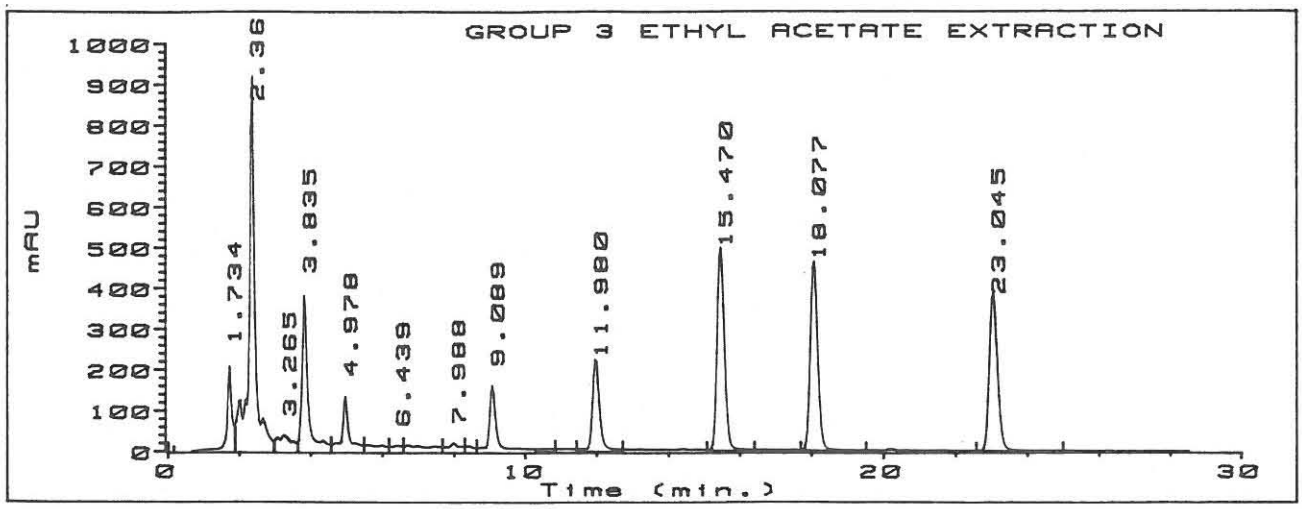


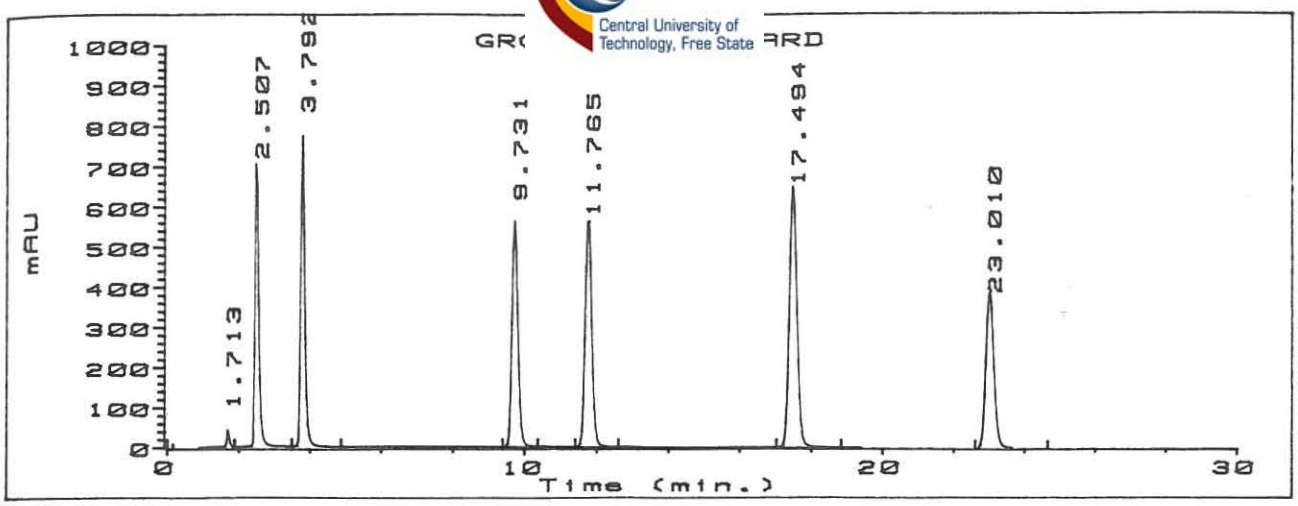


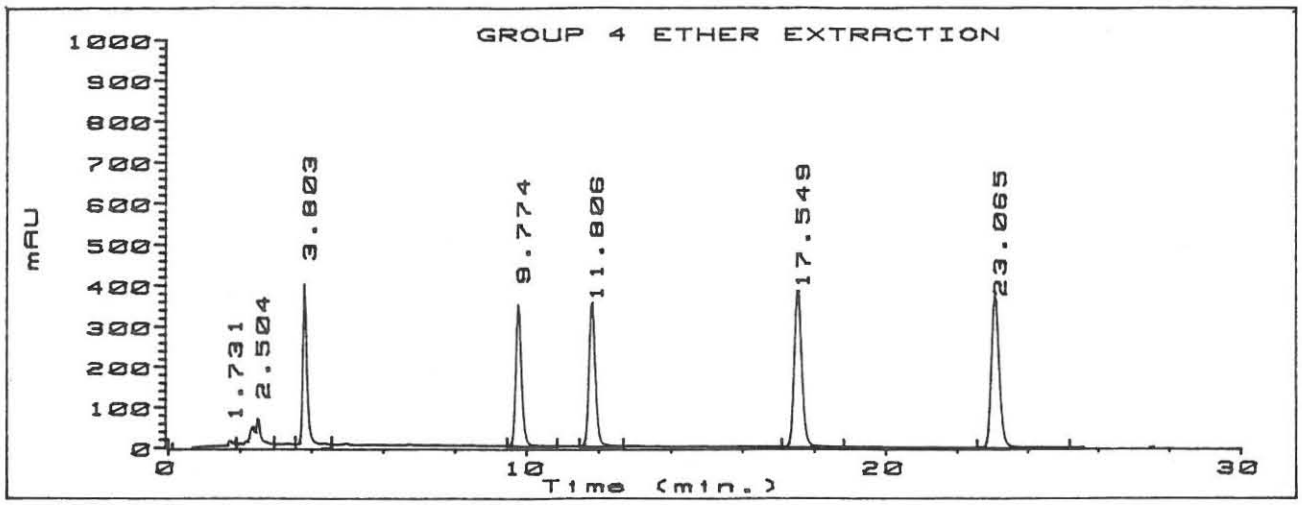


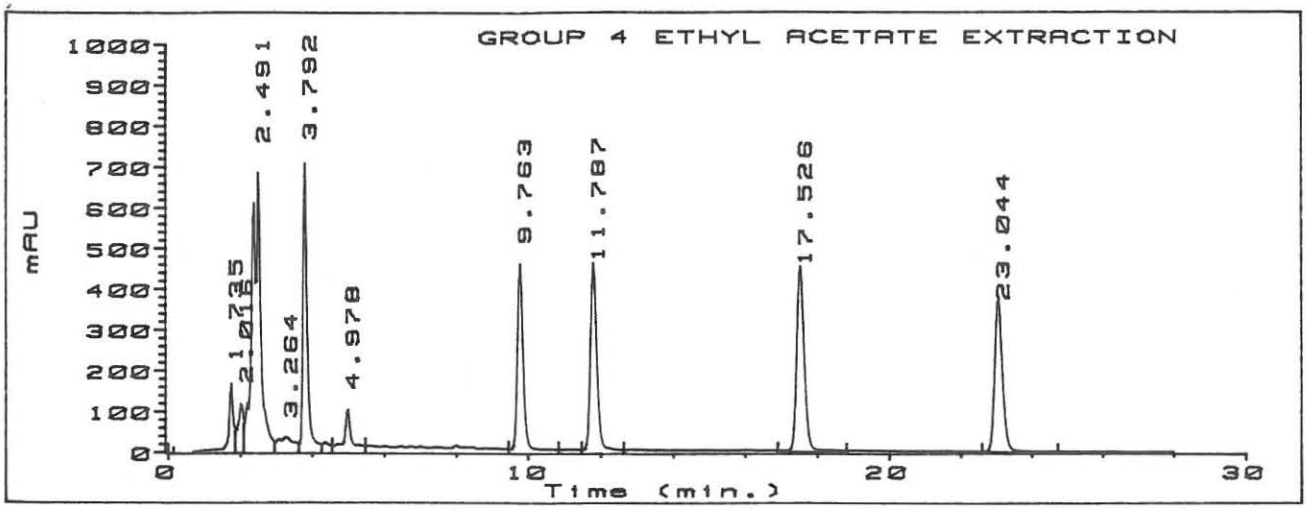


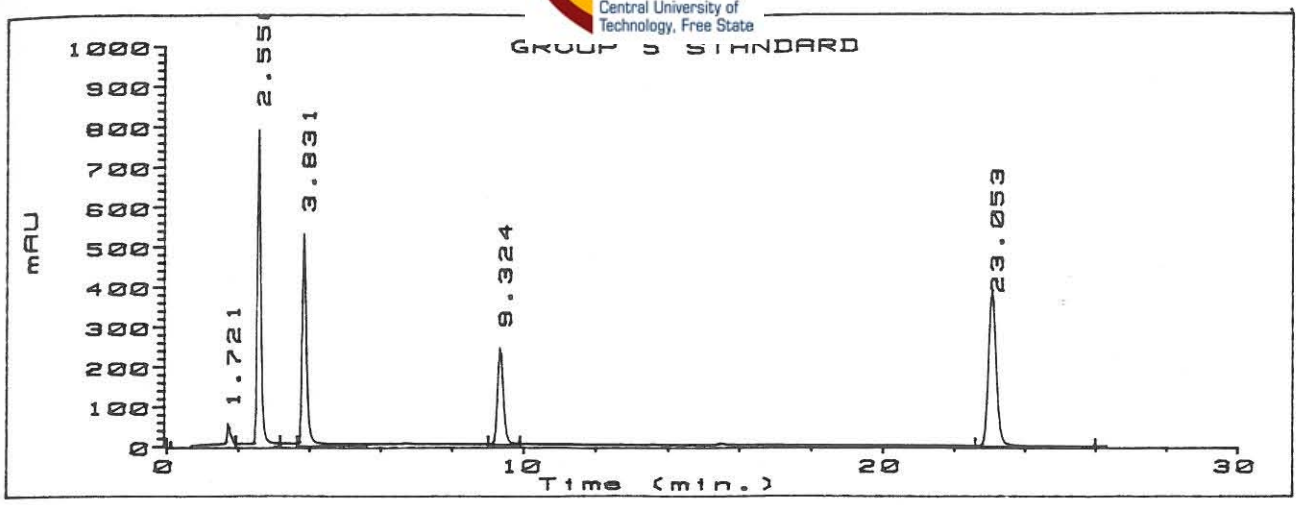


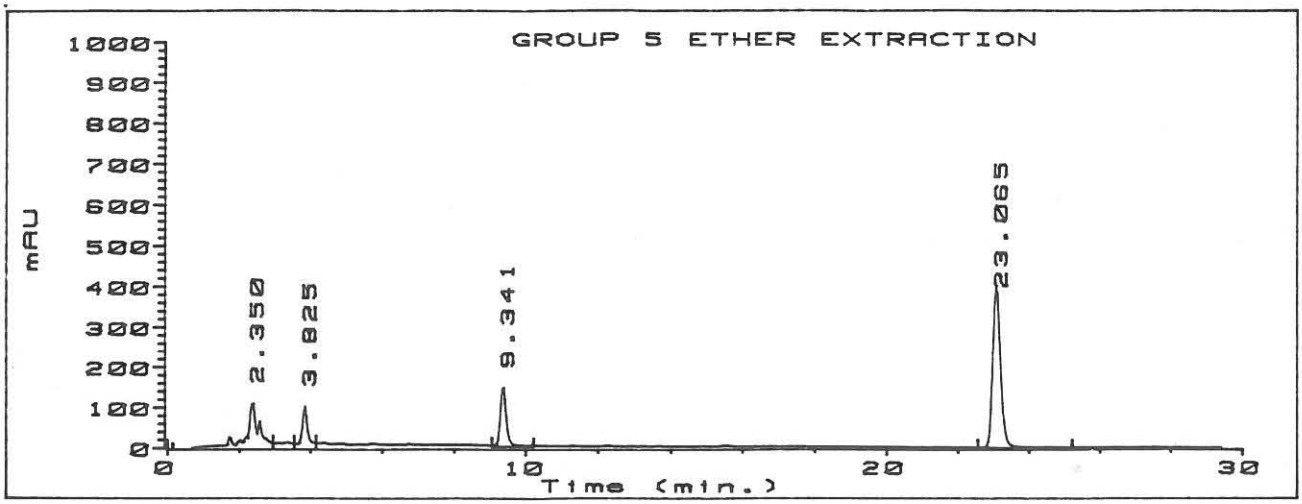


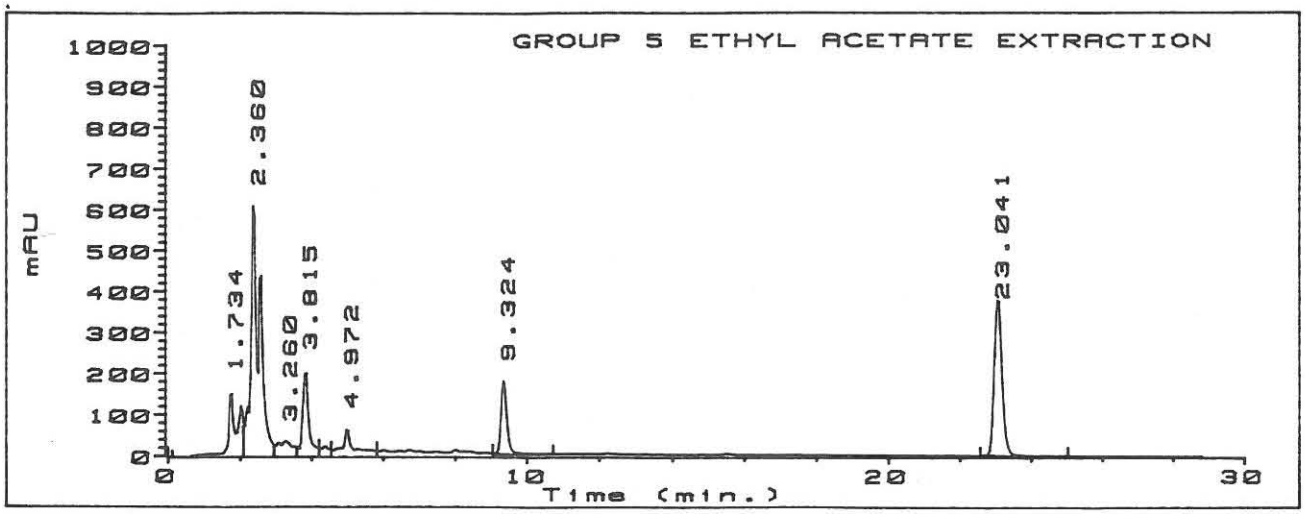


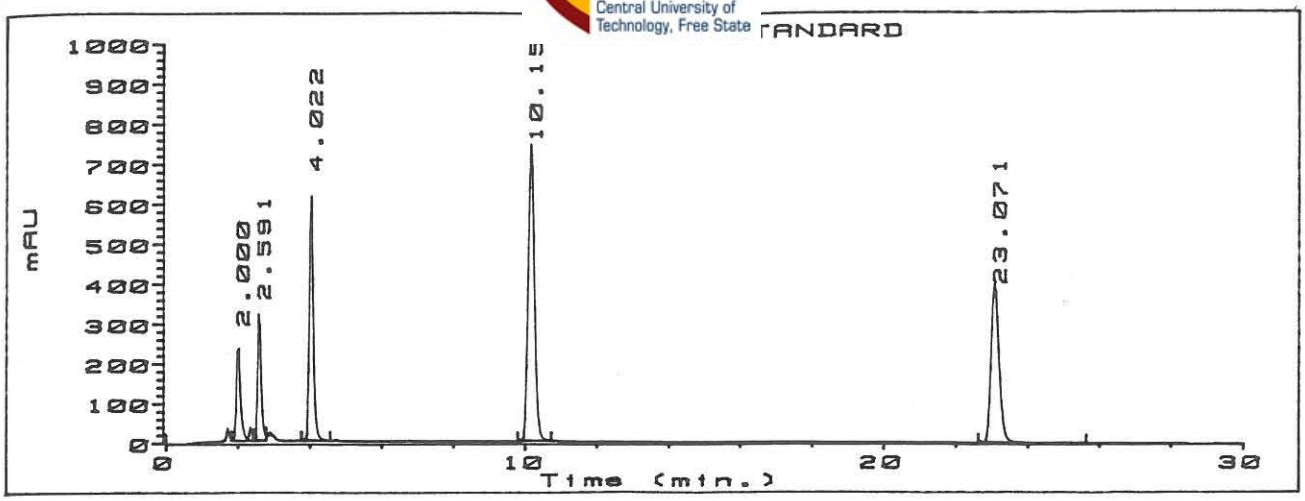


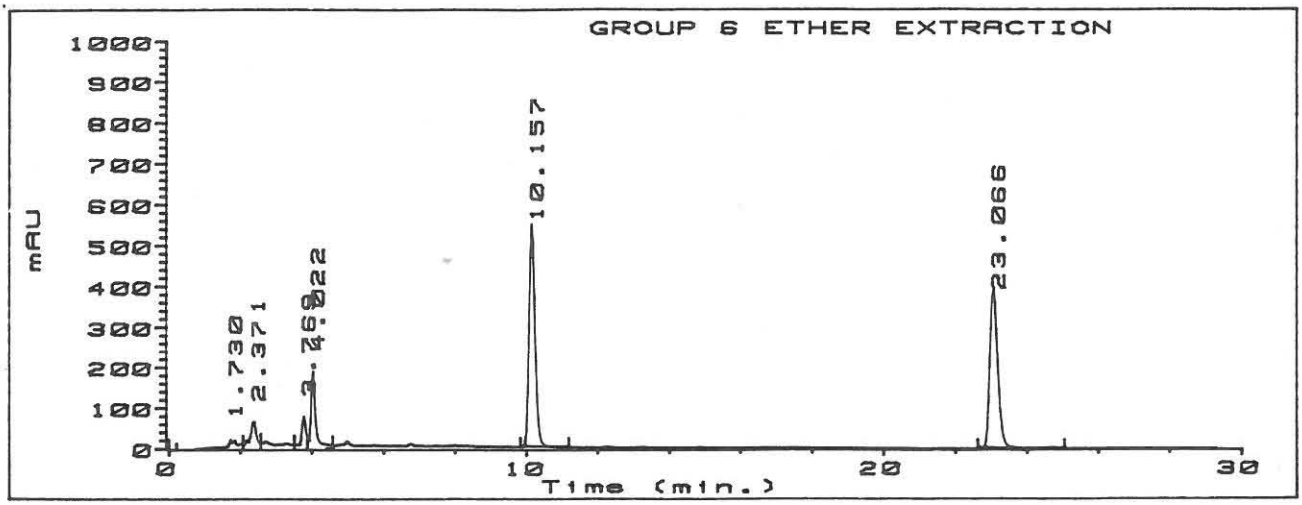


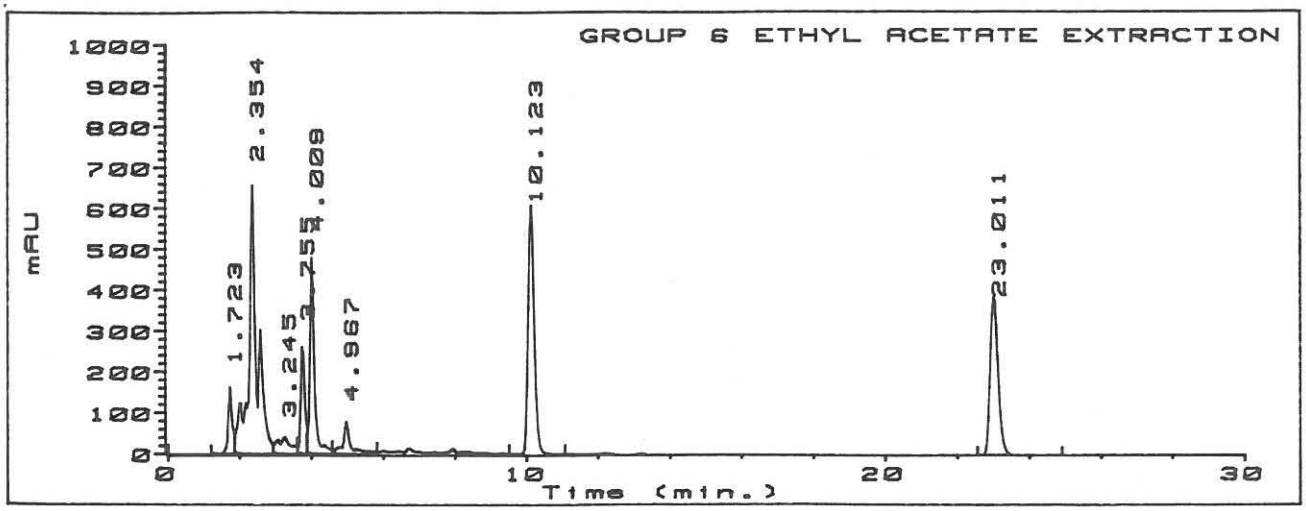


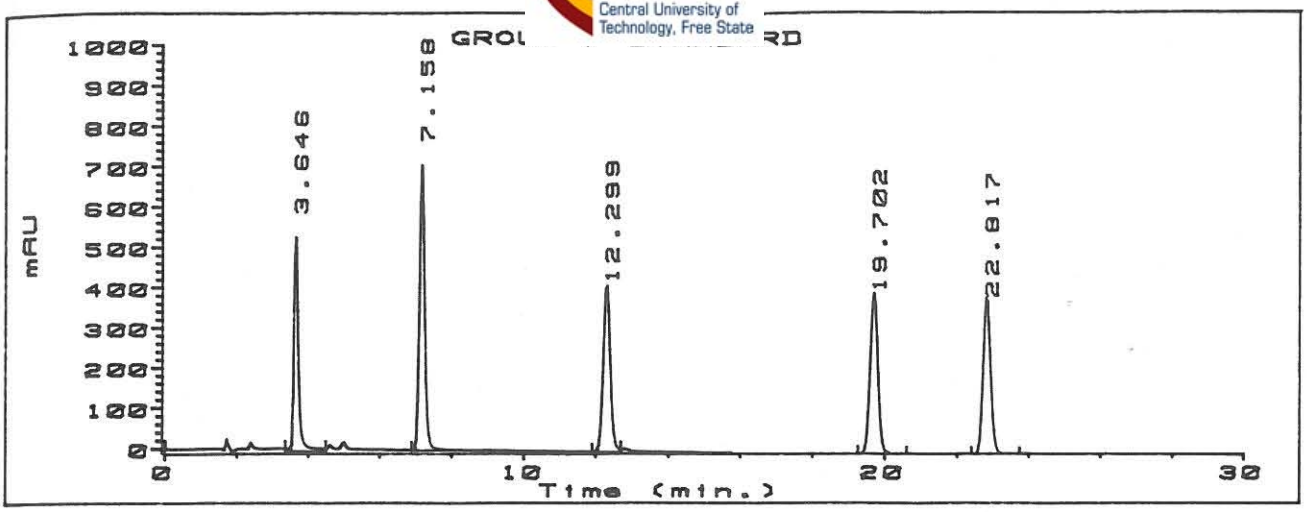


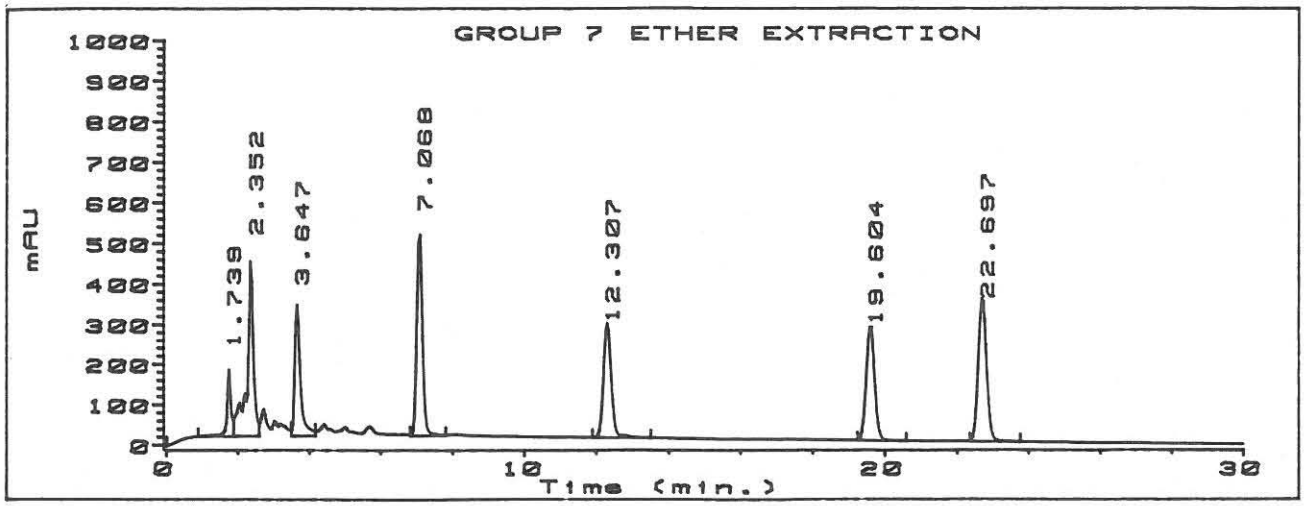


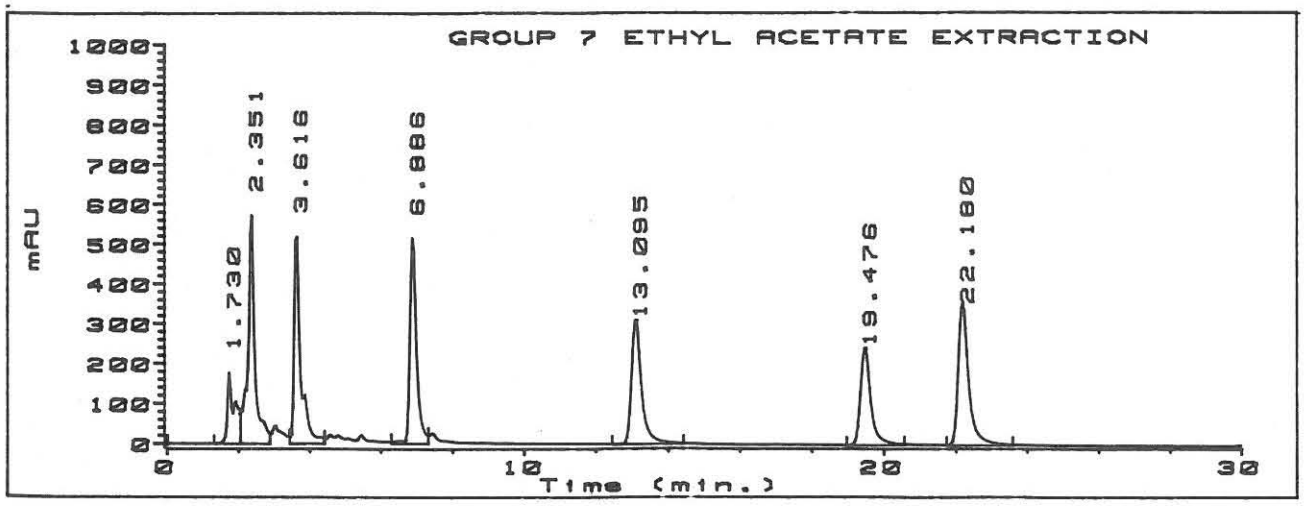


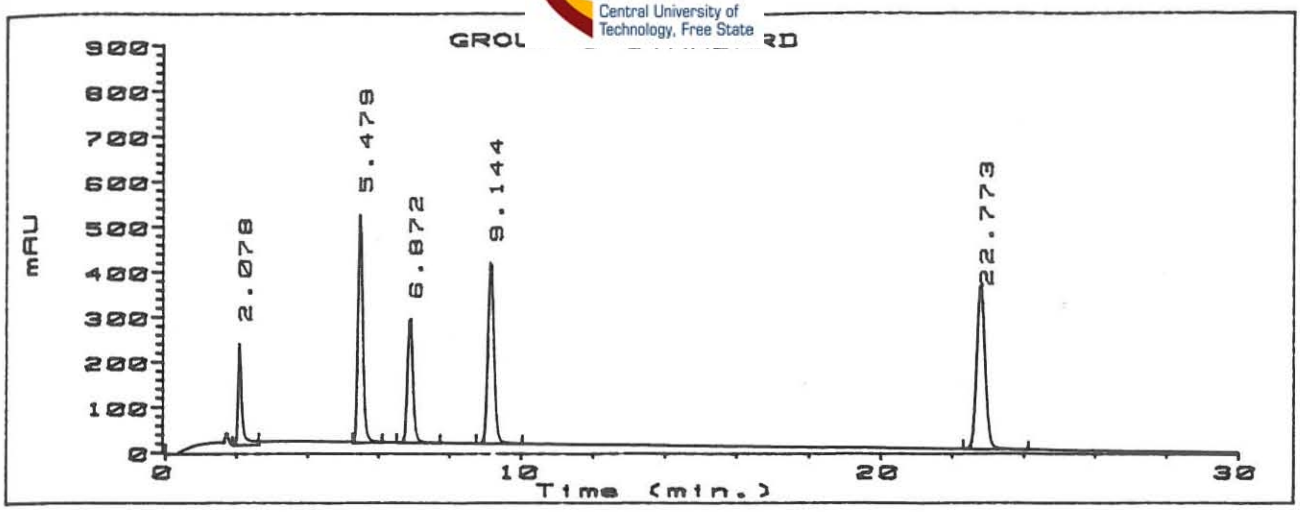


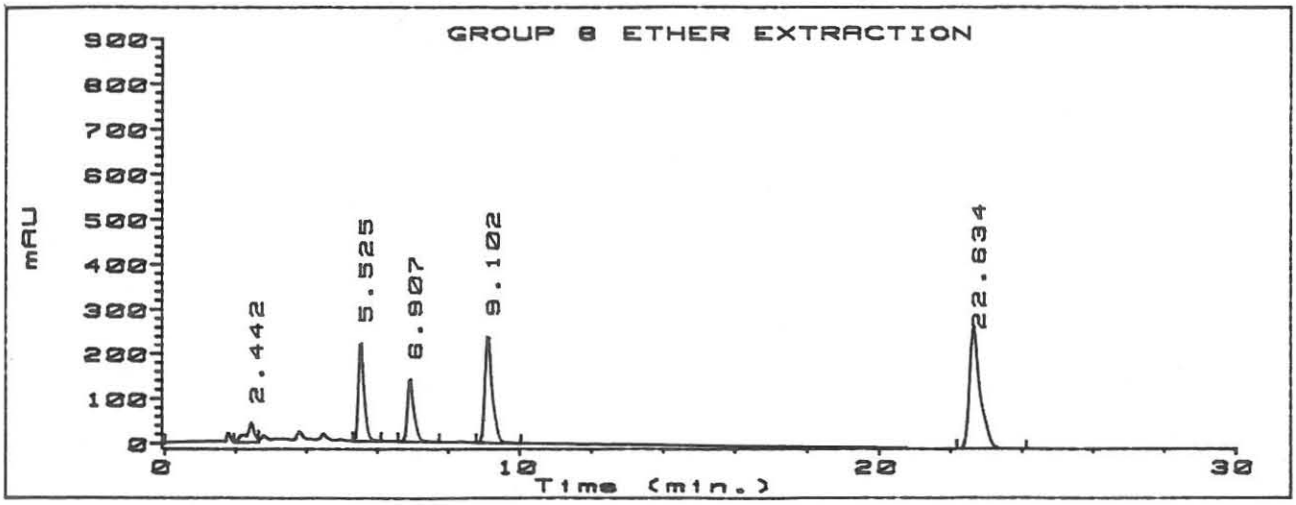


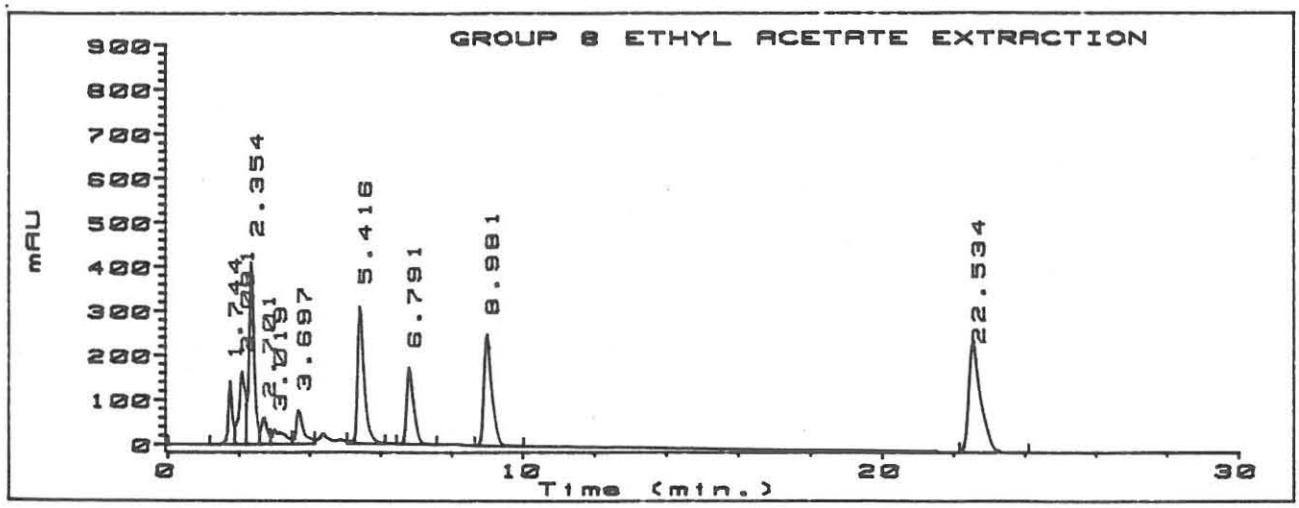


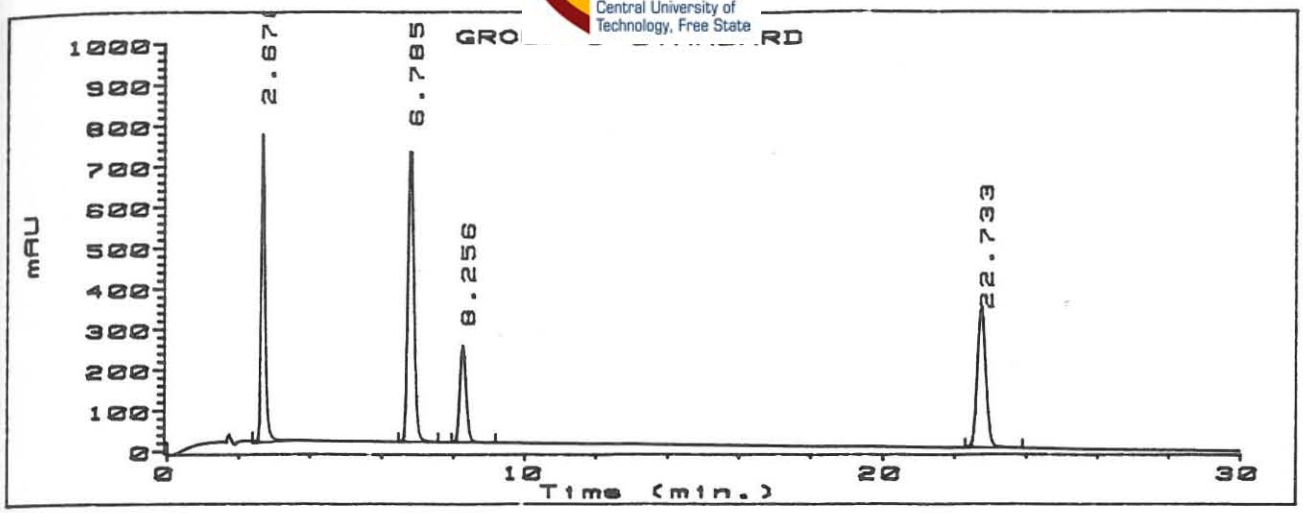


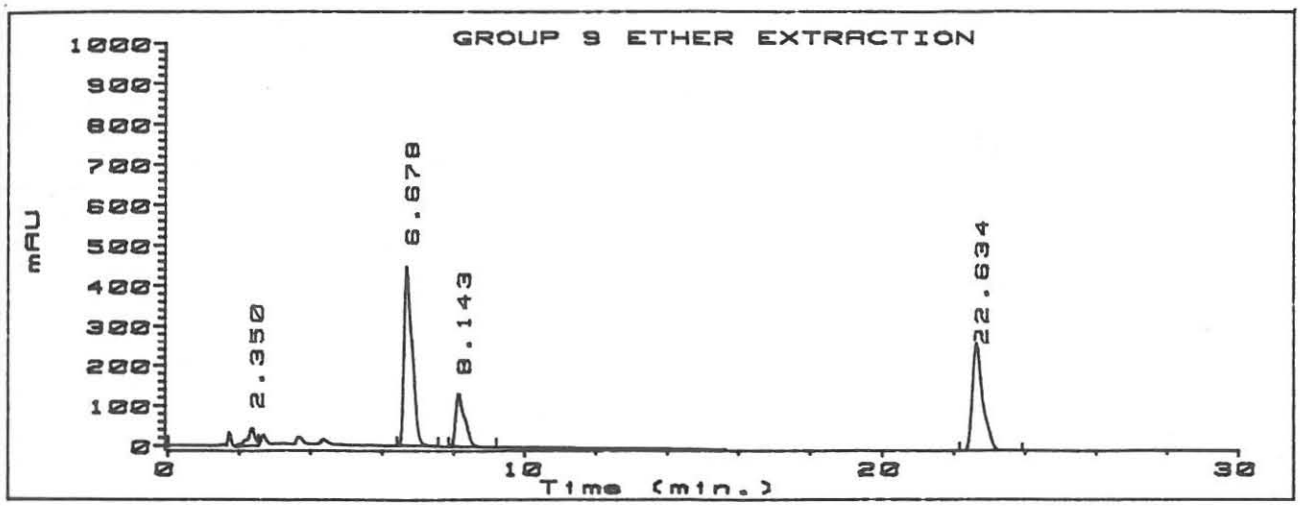


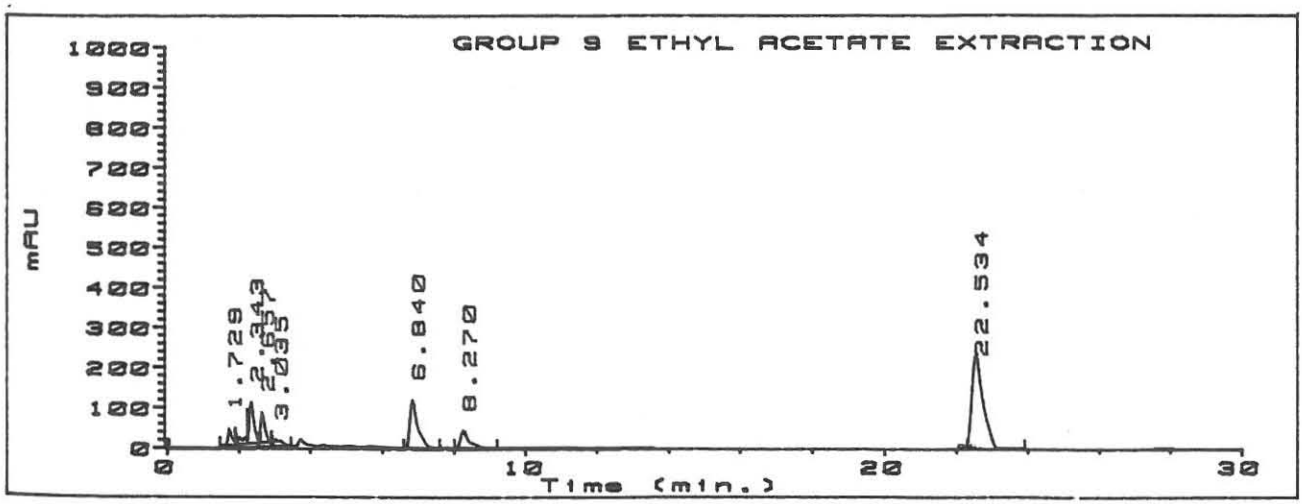


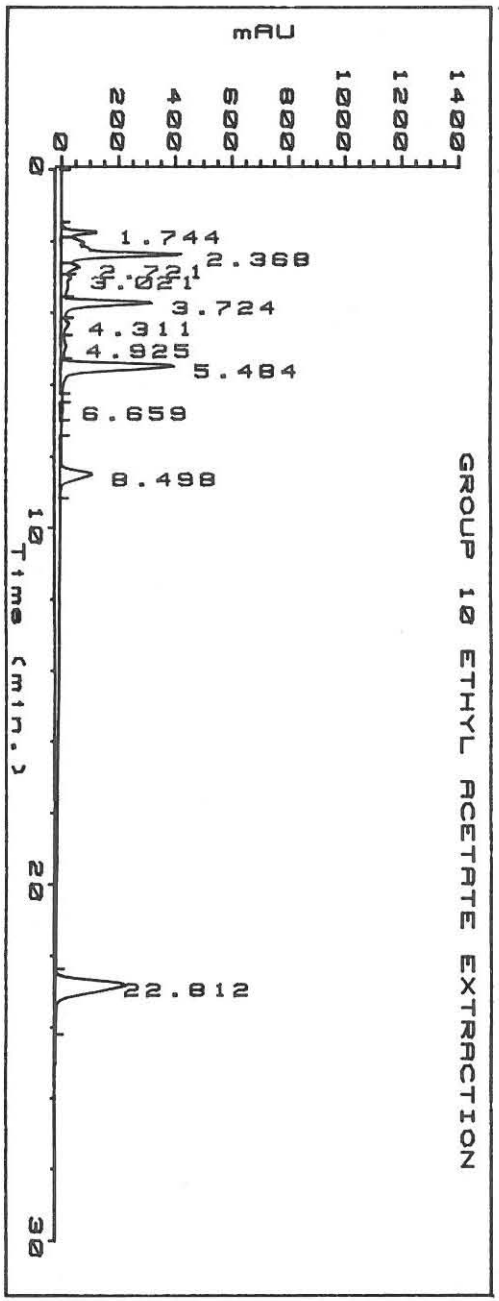


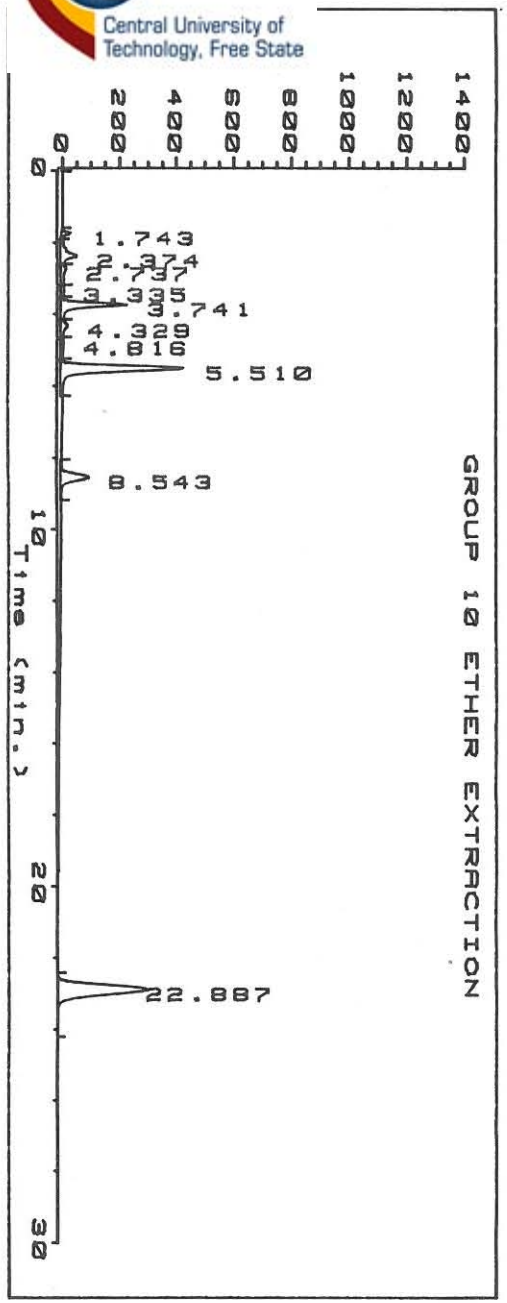


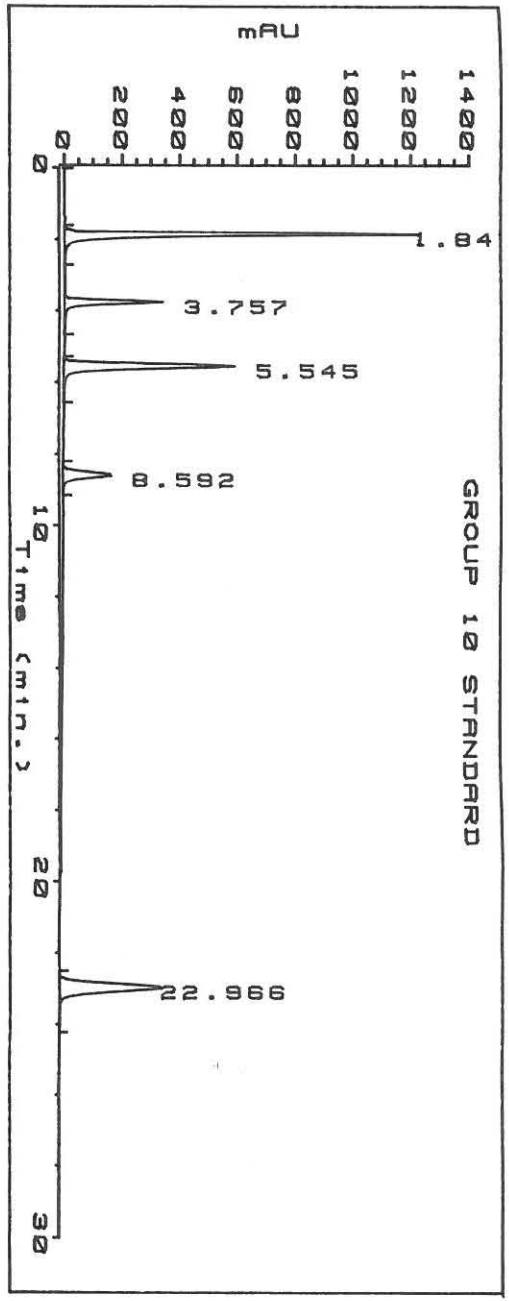


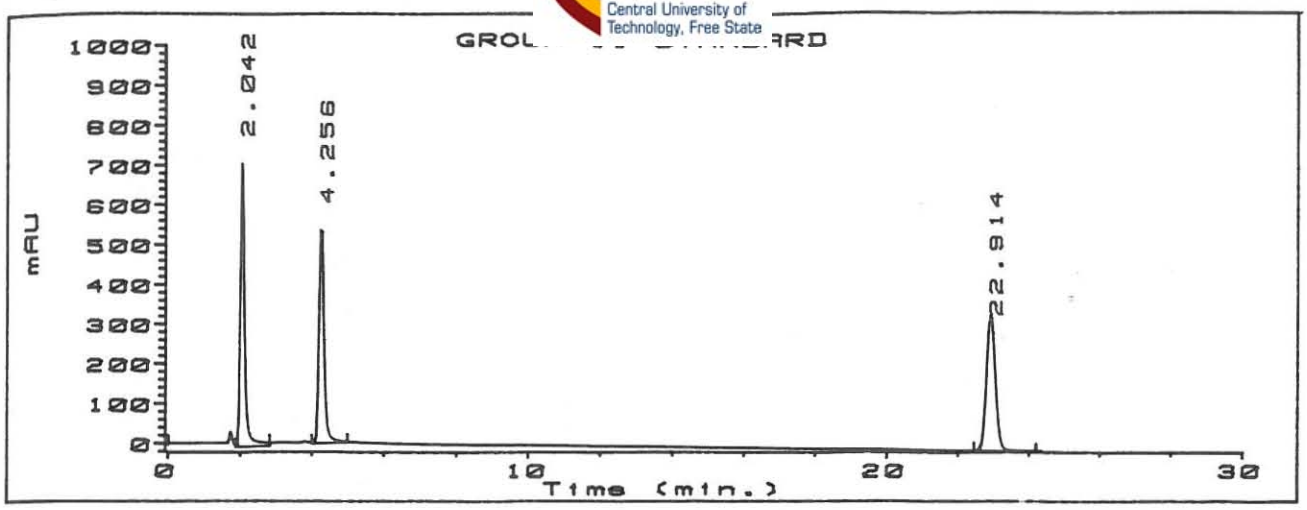


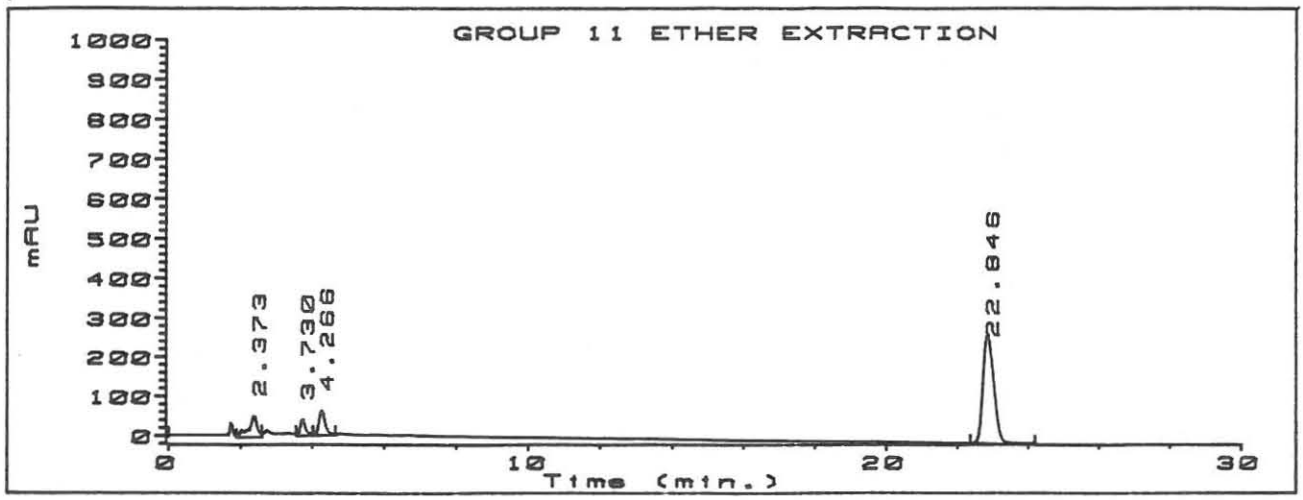


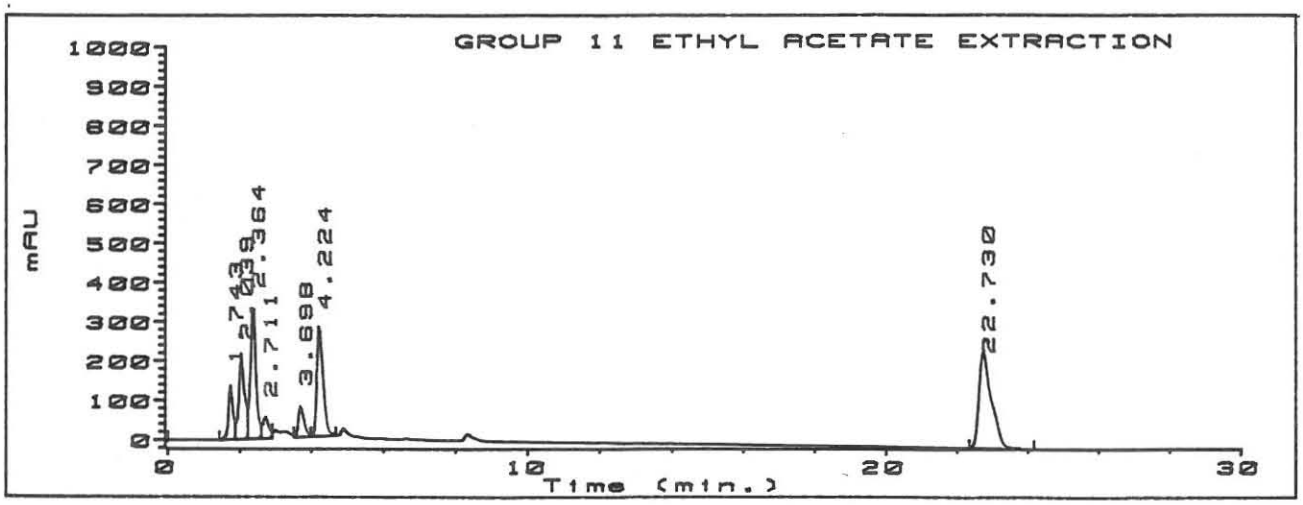


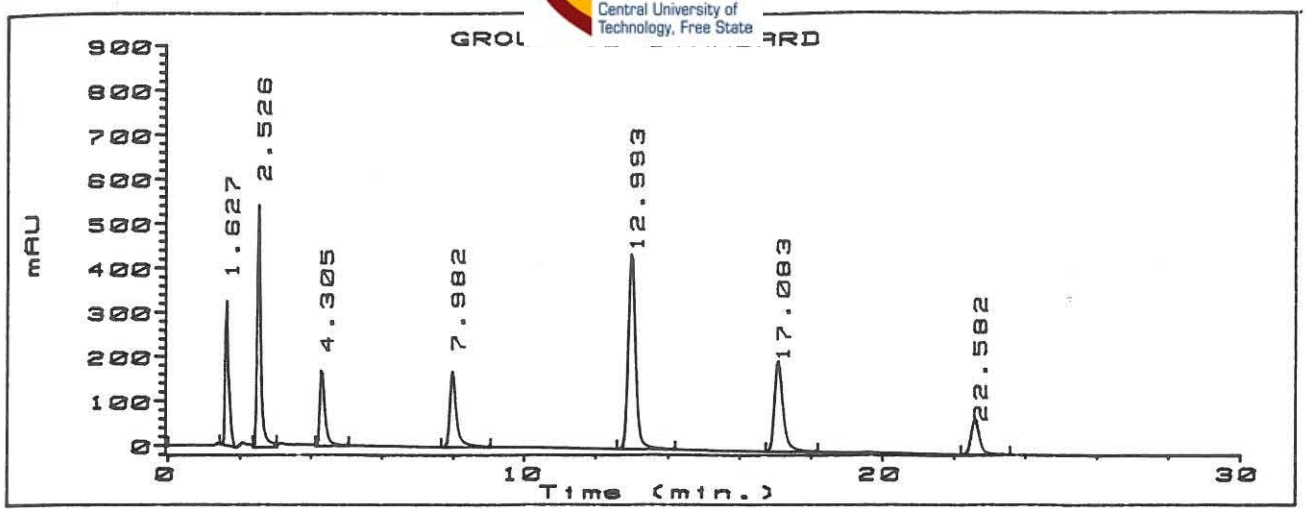


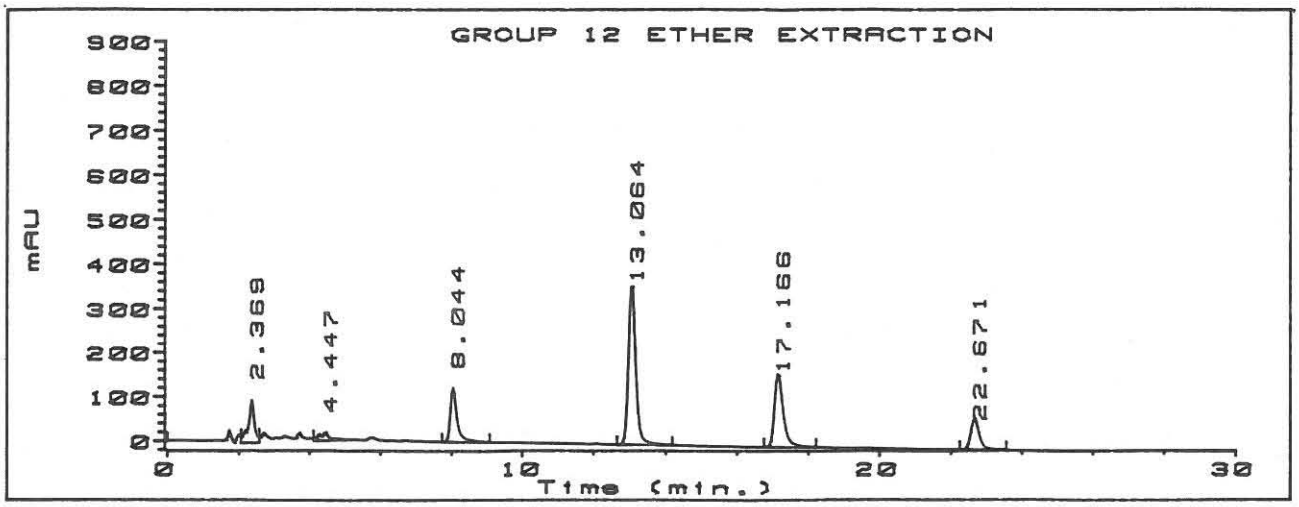


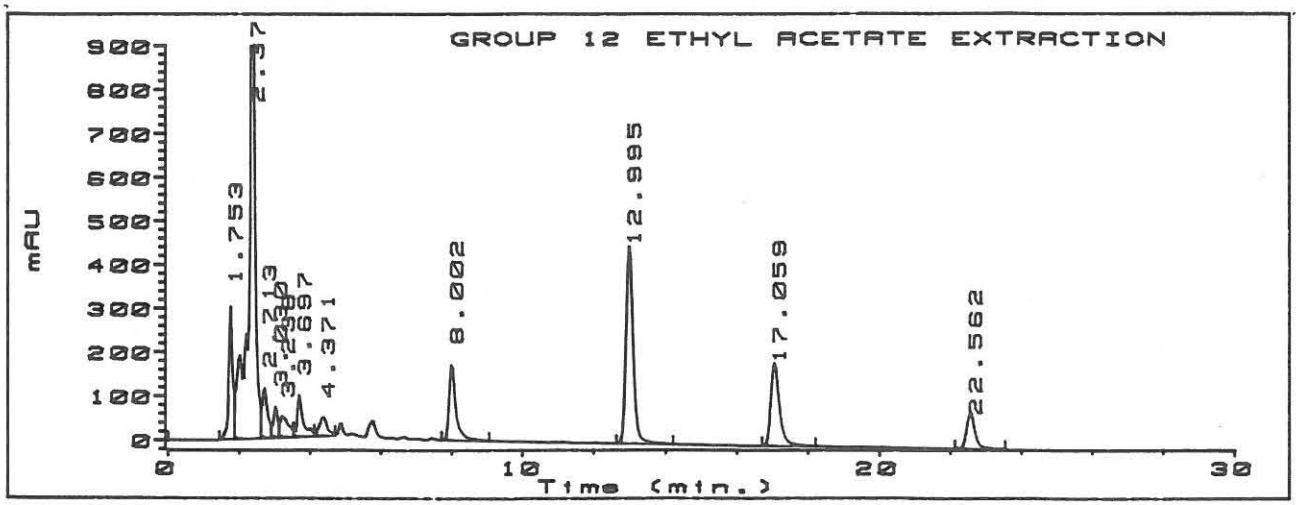


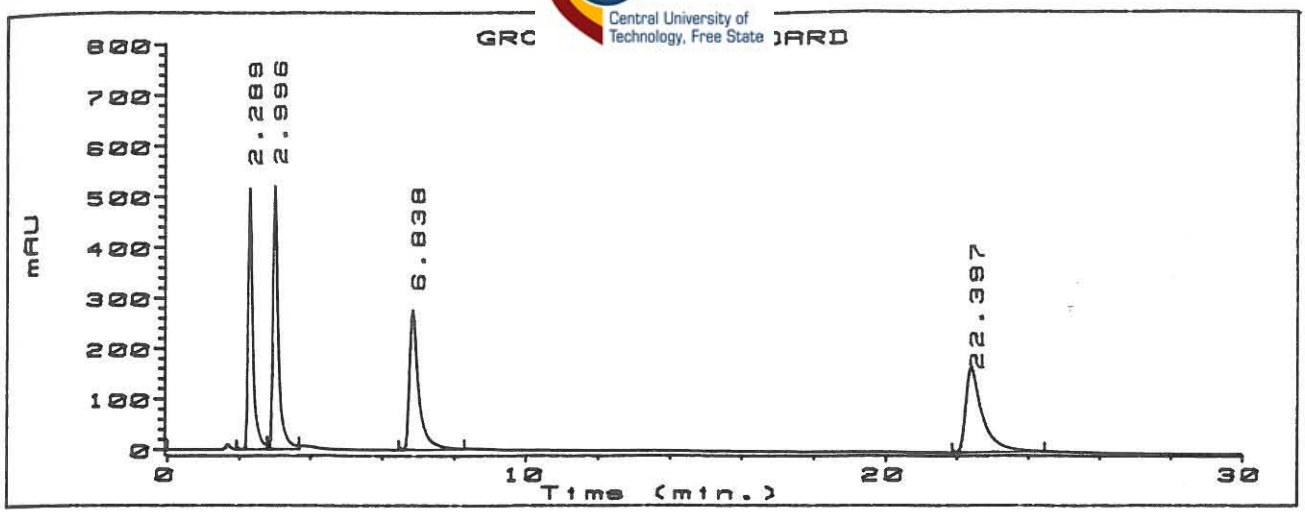


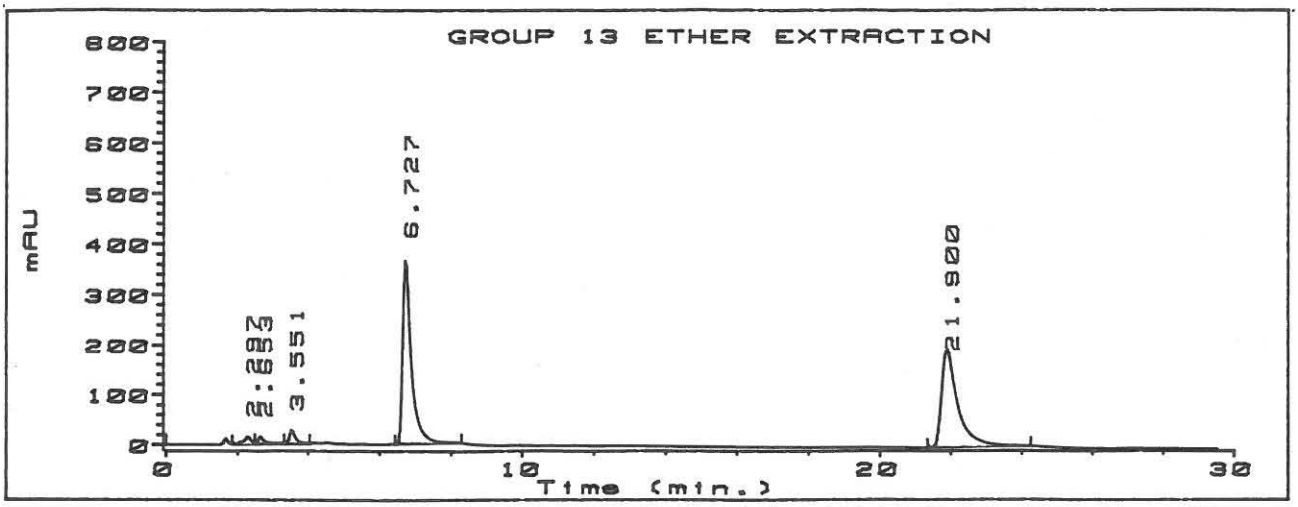


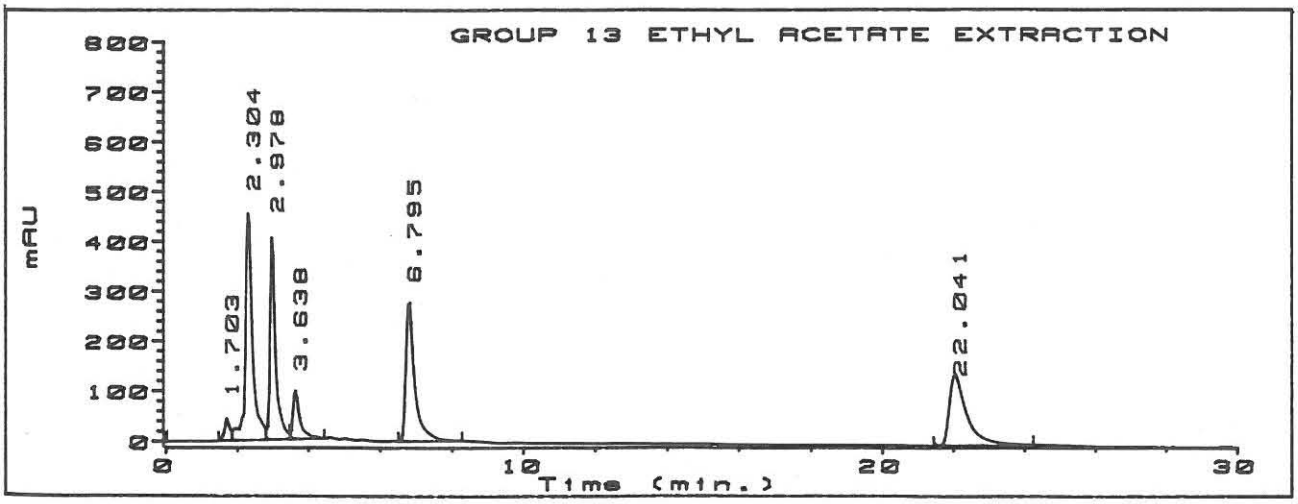


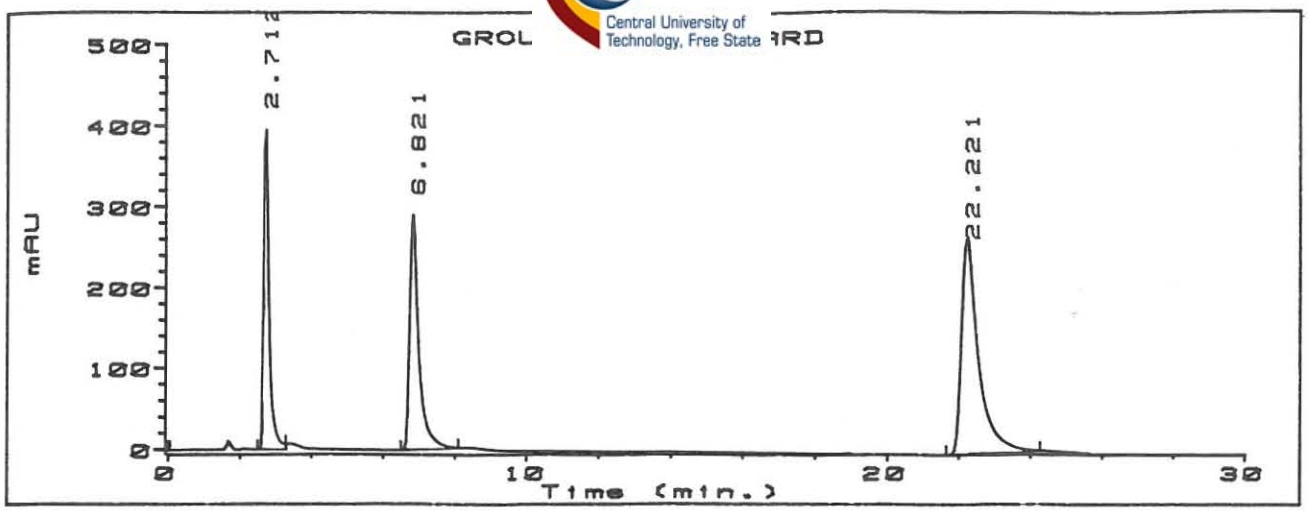


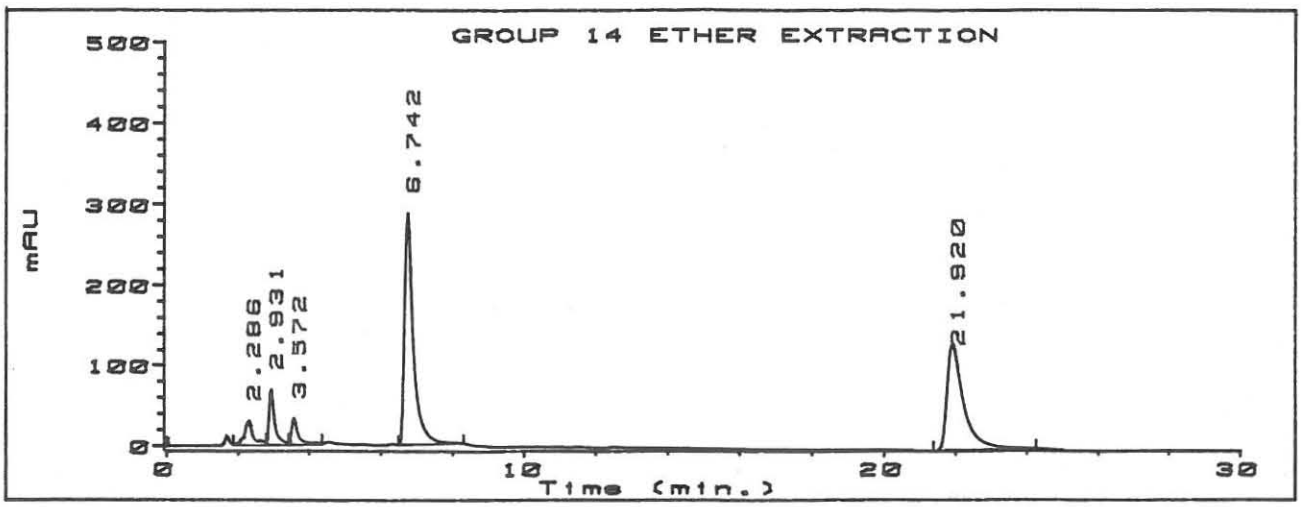


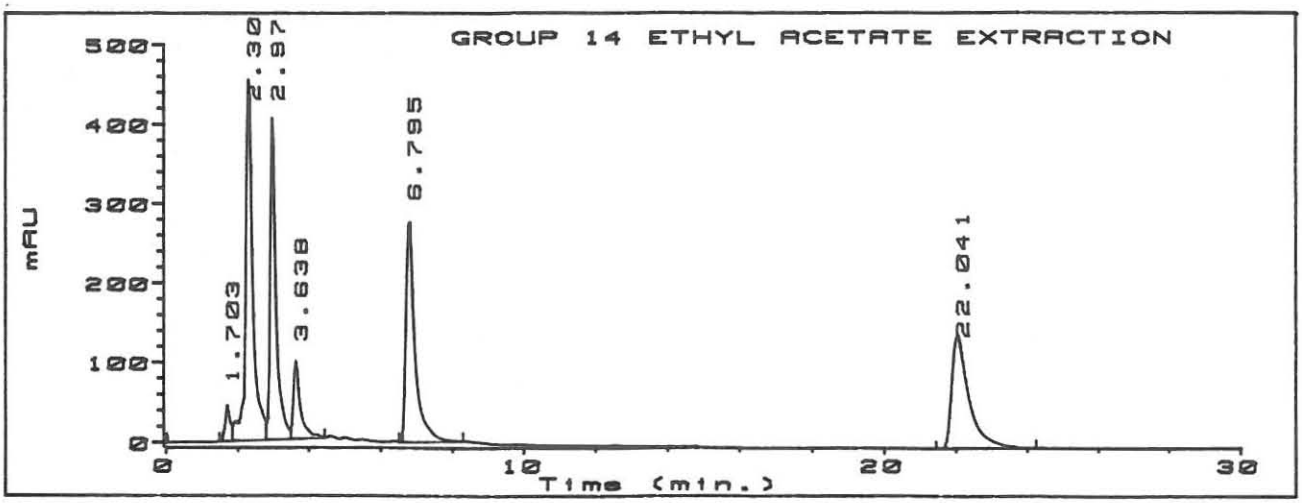




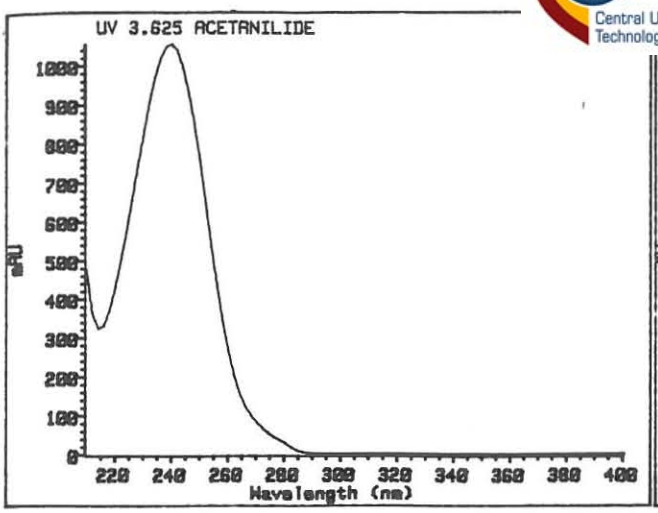


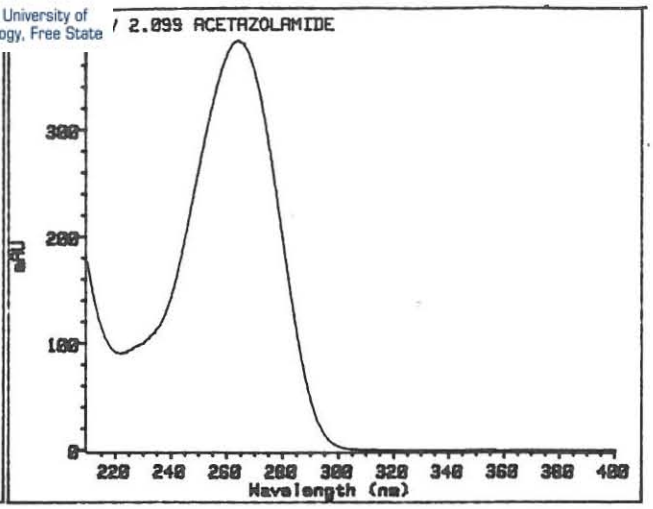


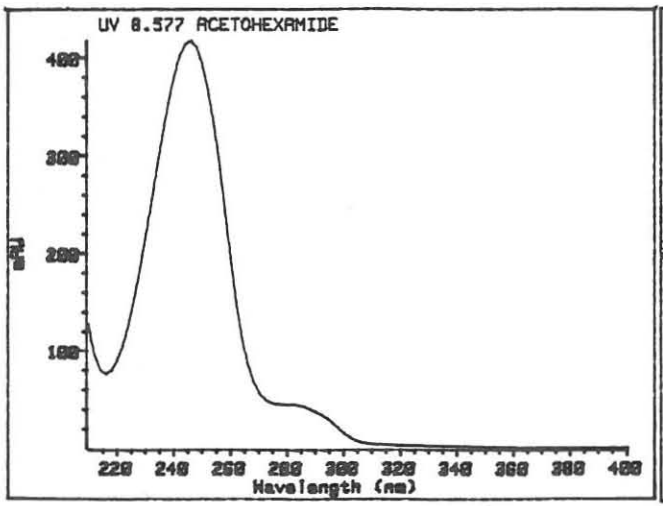


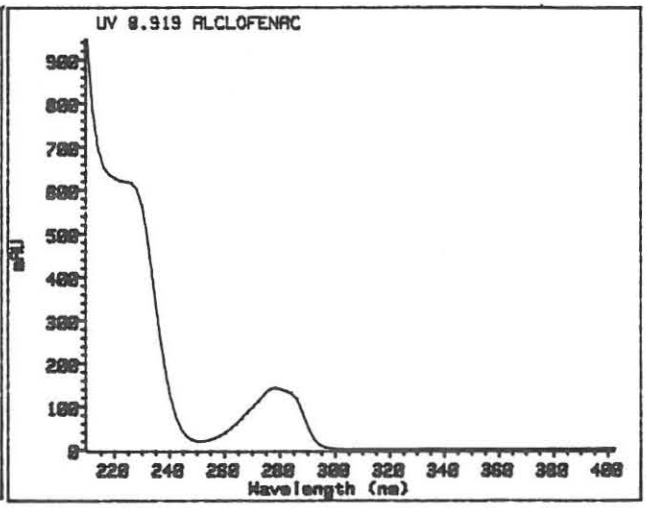


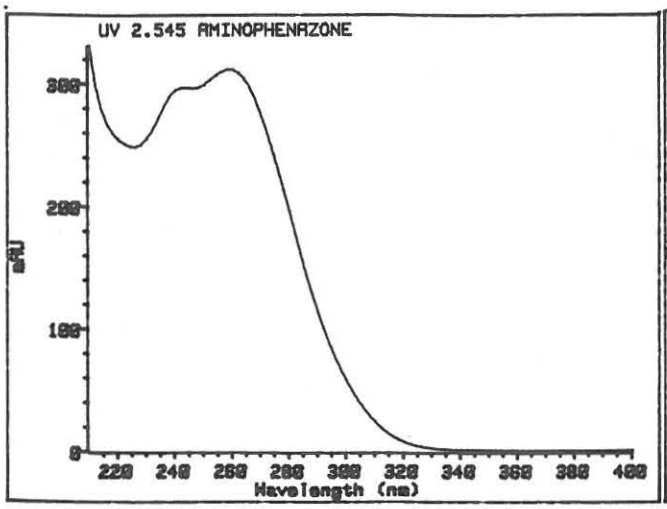
APPENDIX II

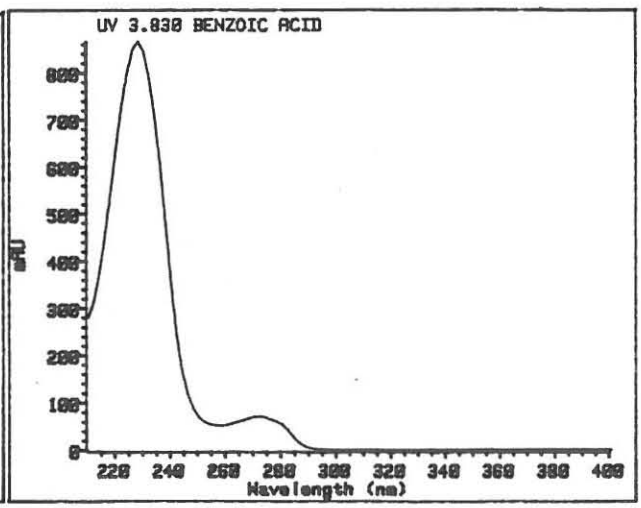


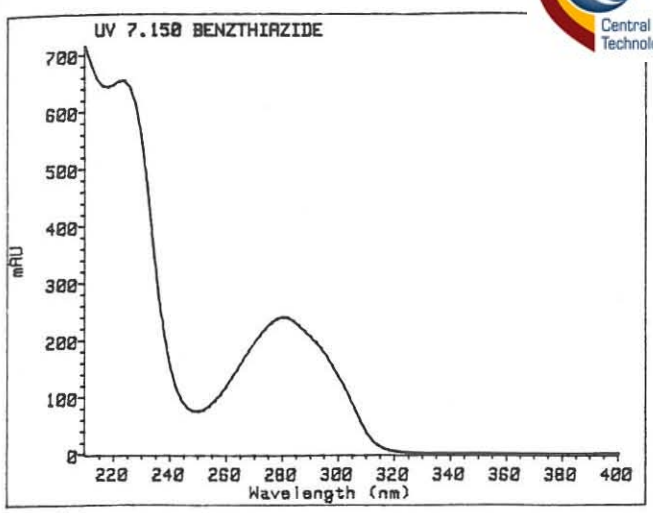


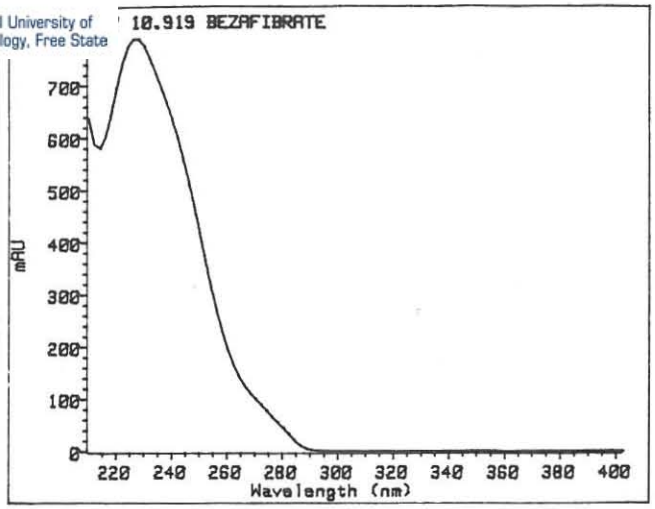


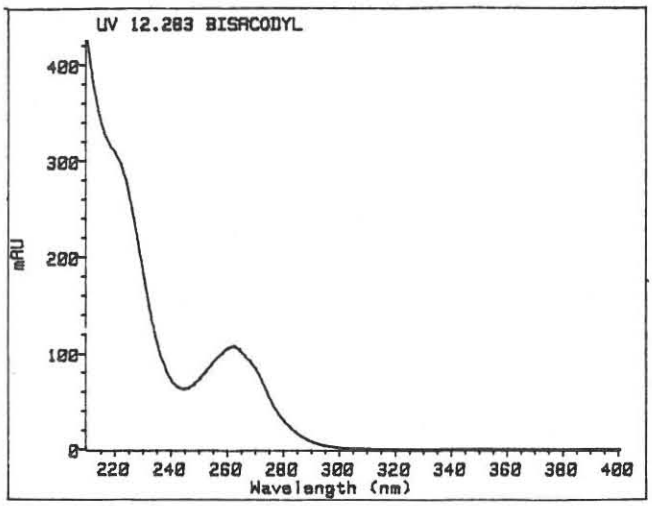


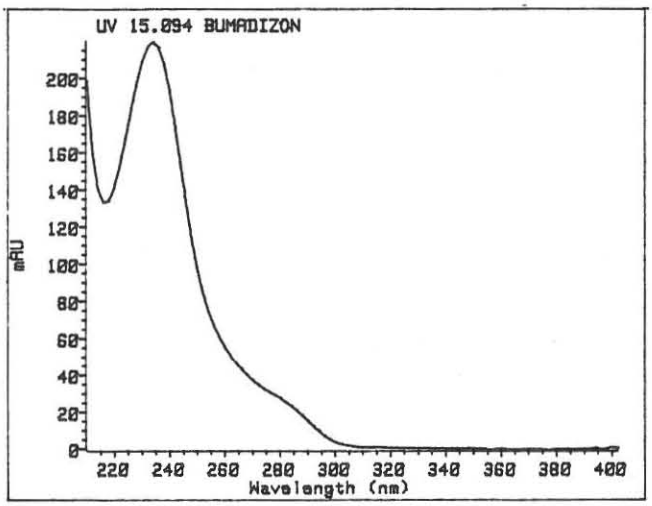


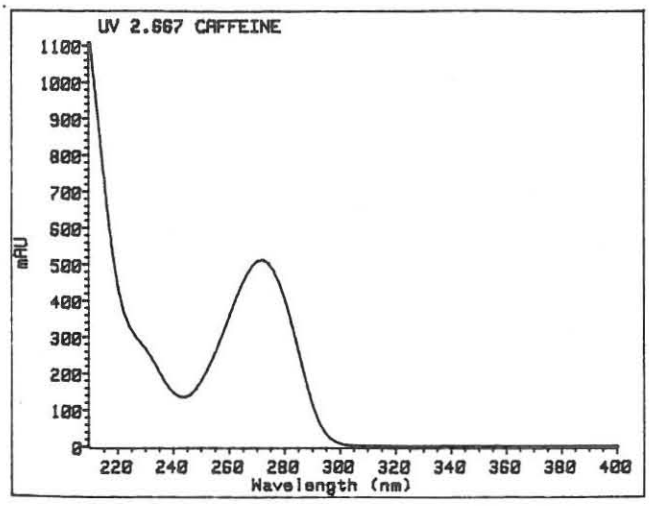


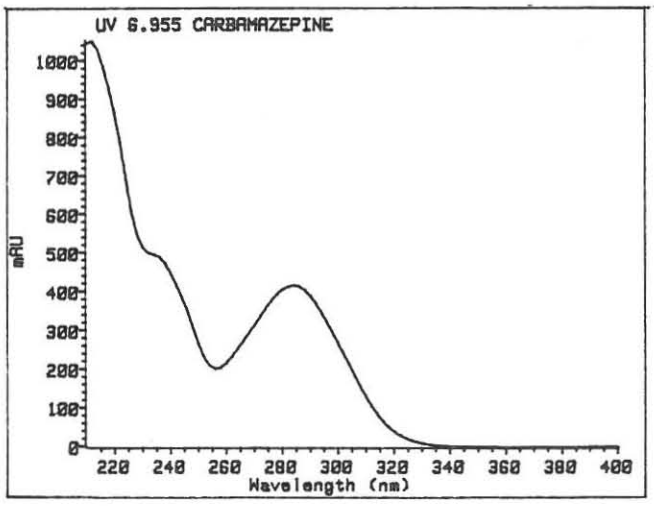


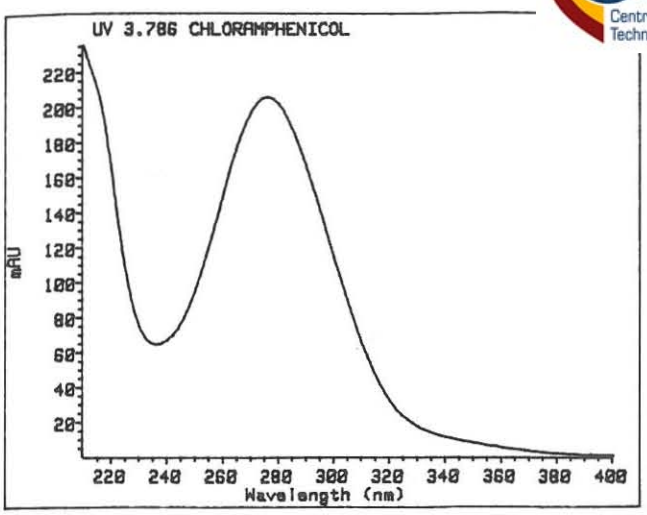


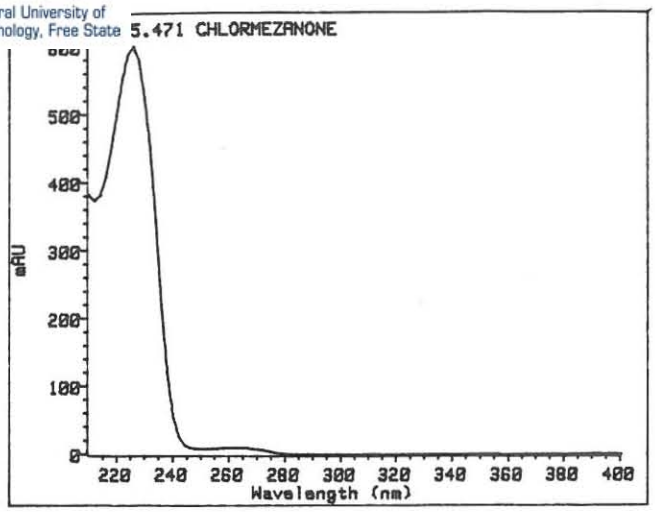


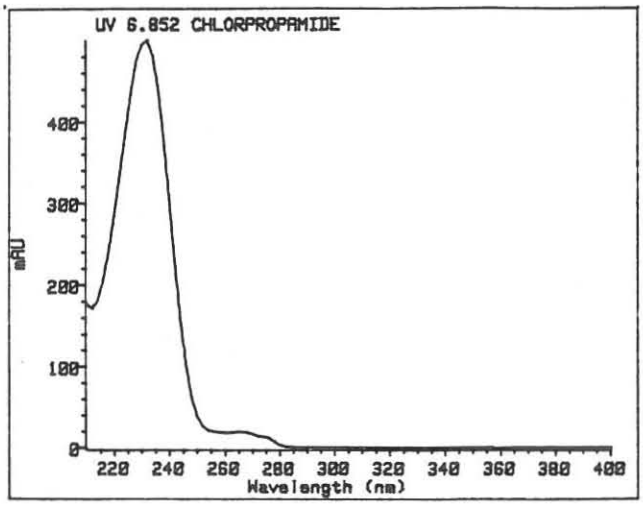


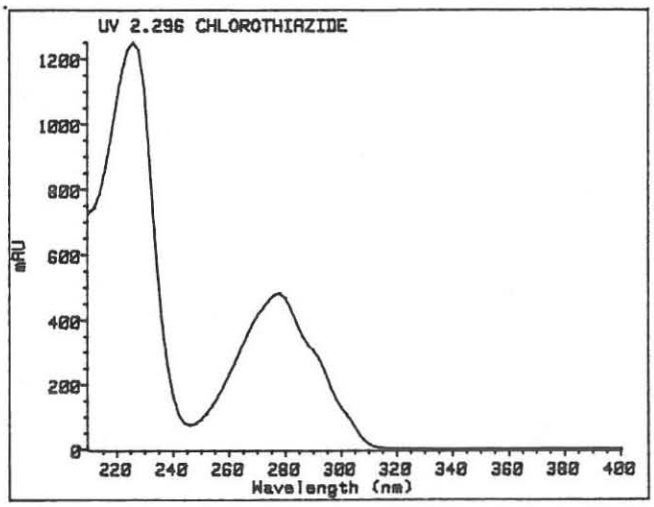


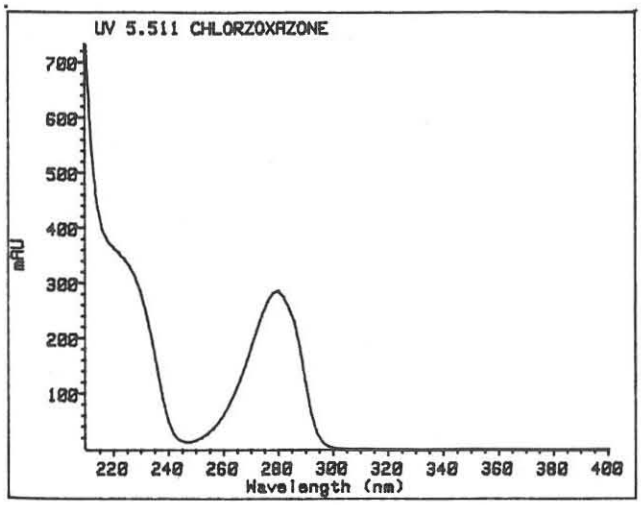


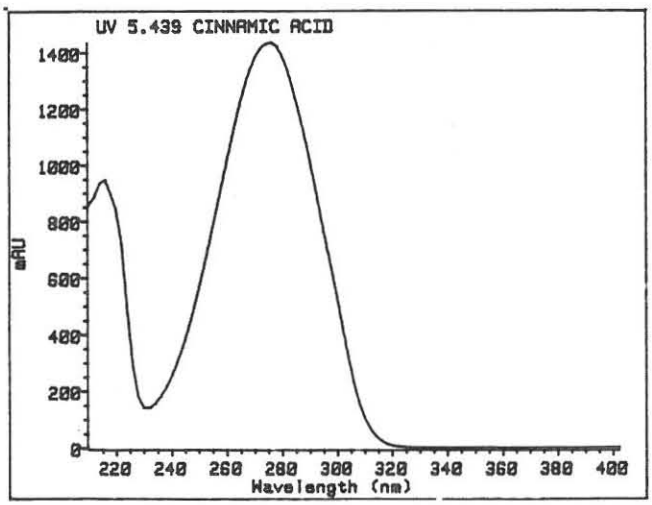


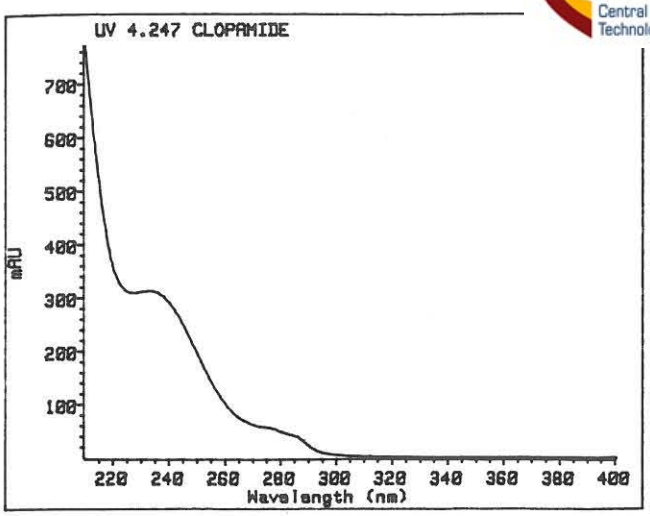


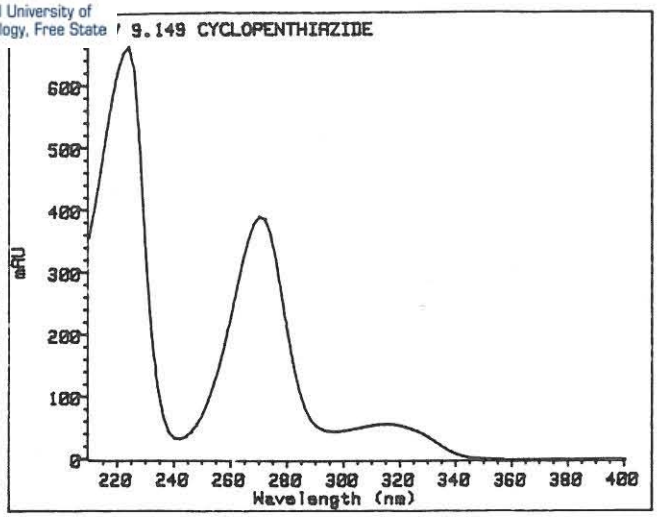


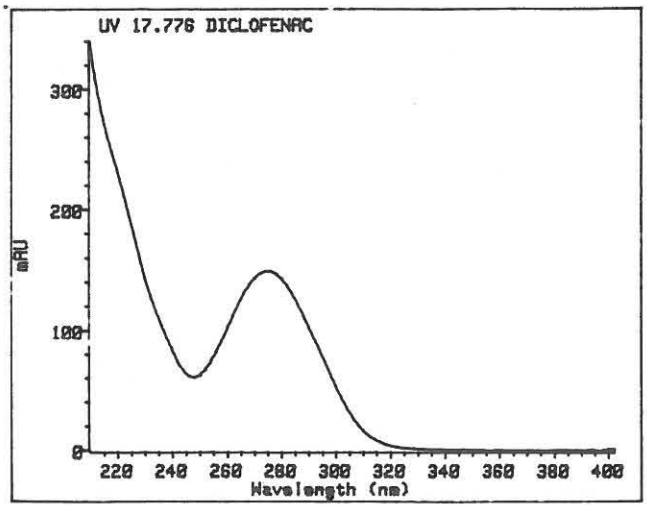


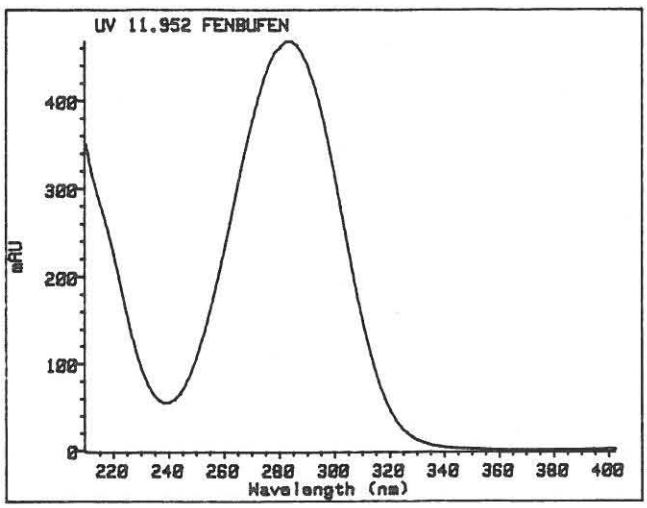


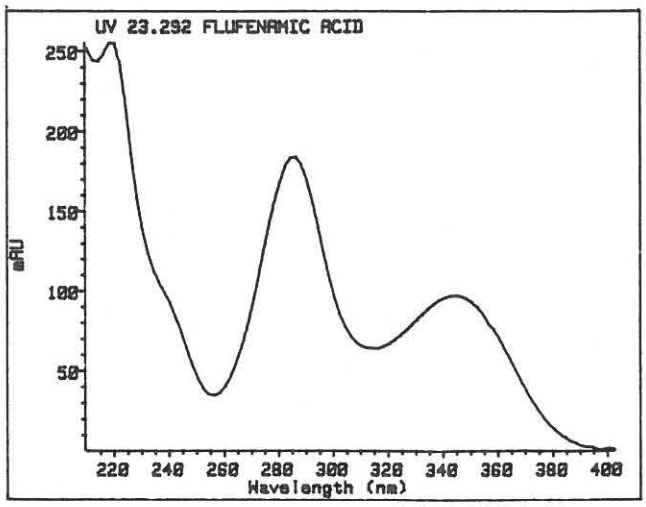


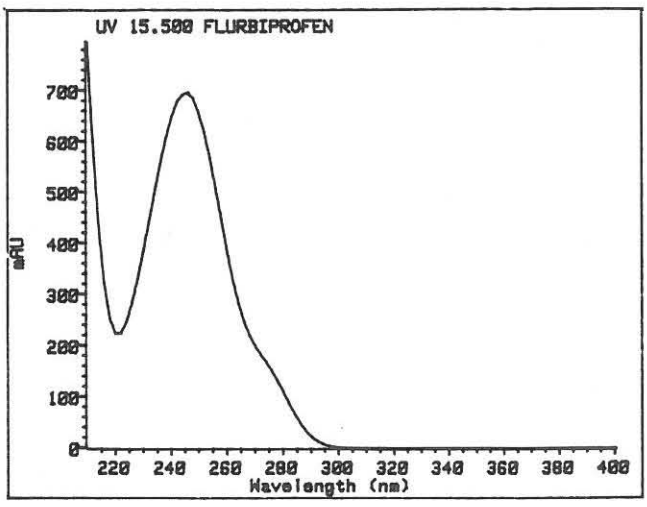


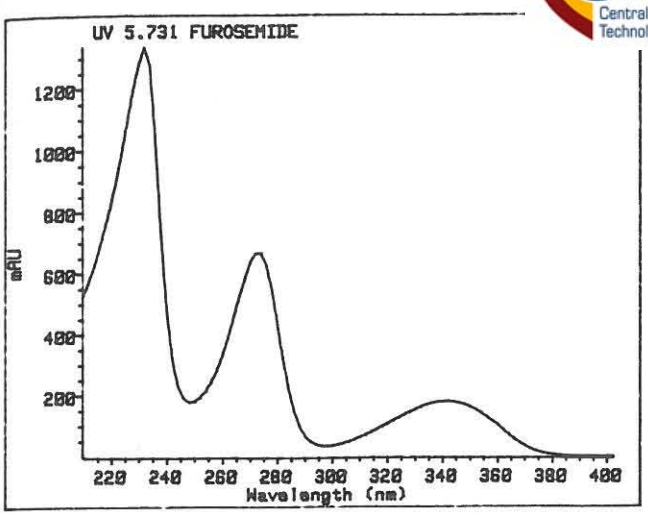


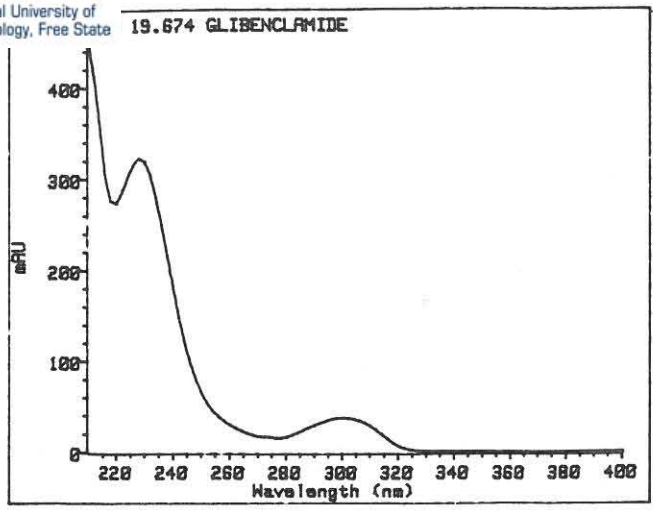


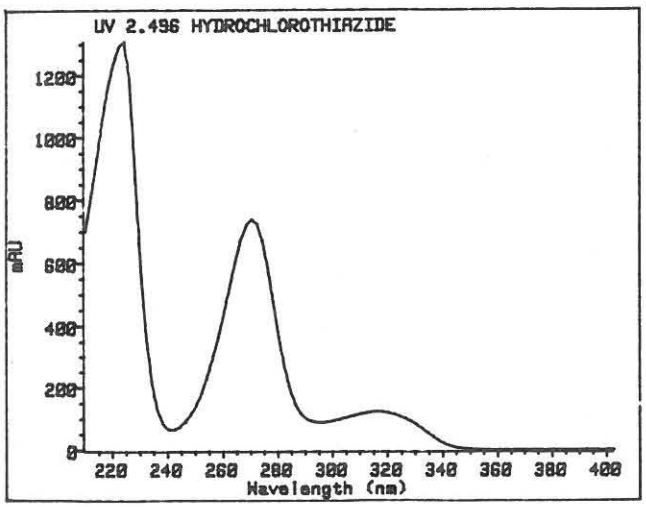


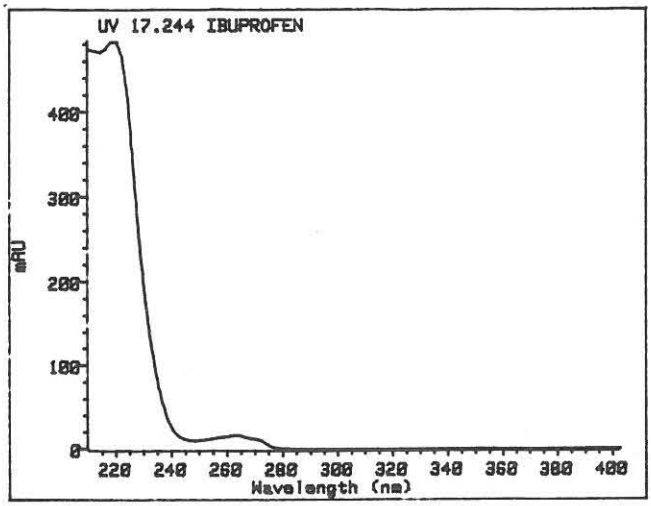


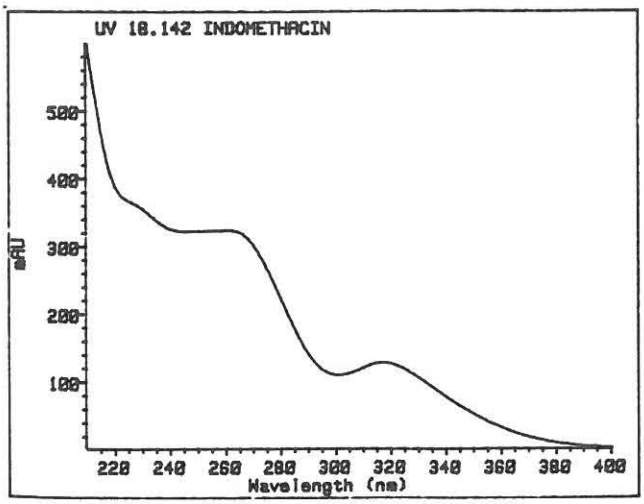


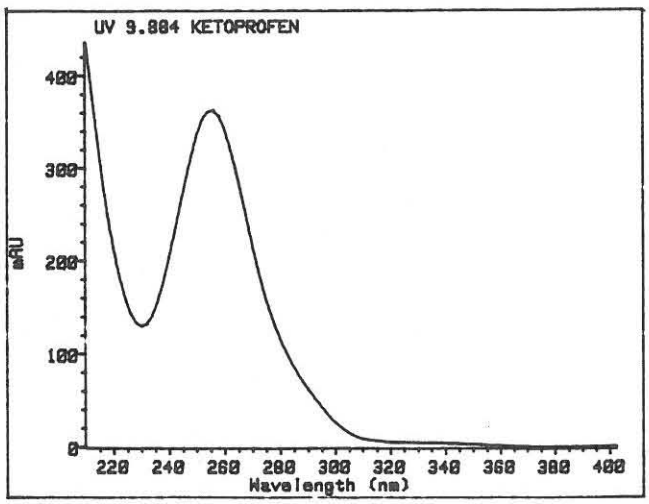


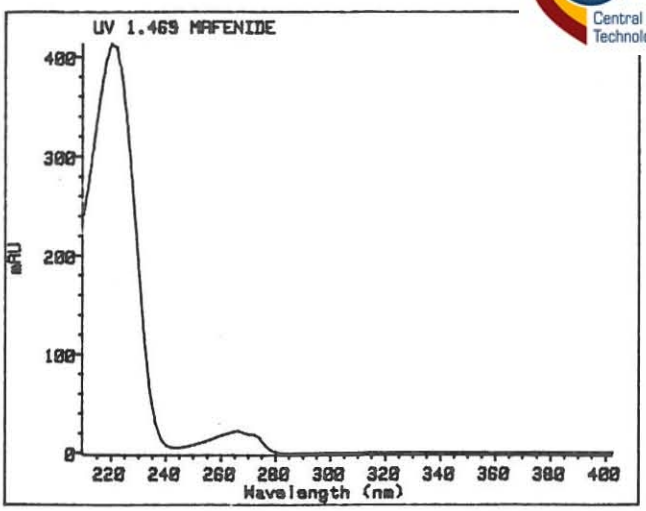


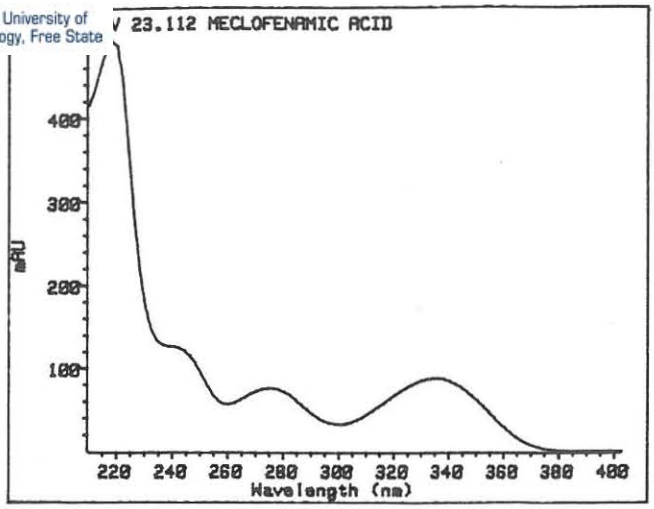


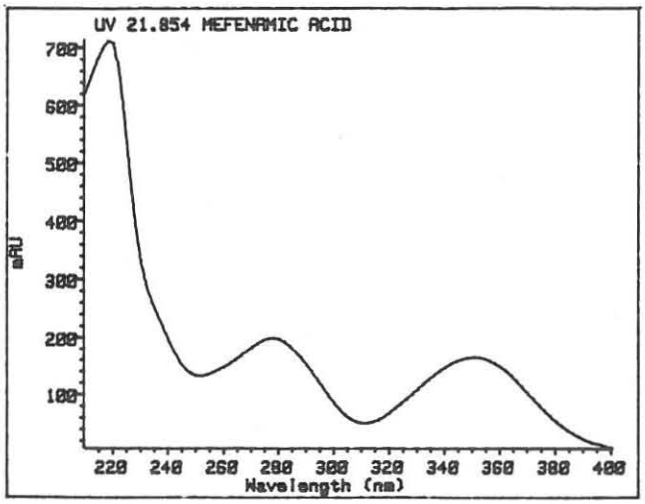


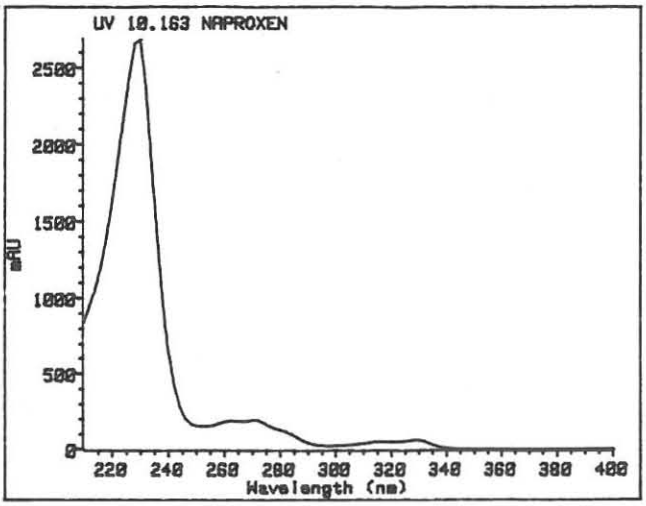


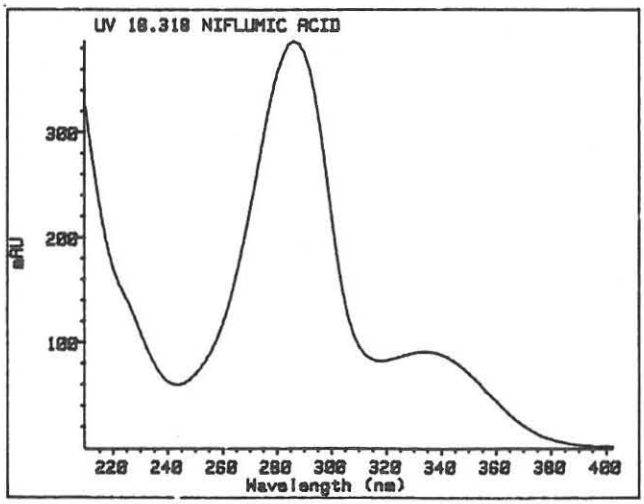


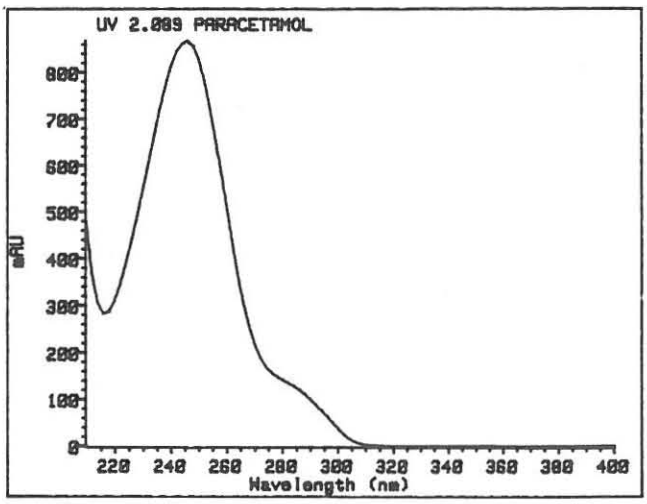


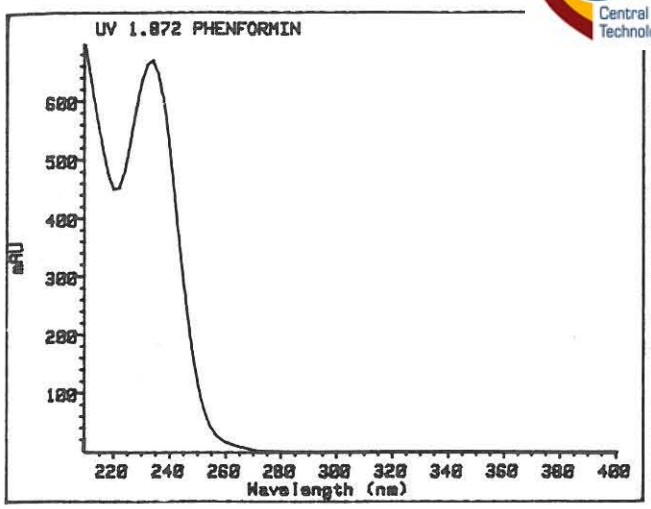


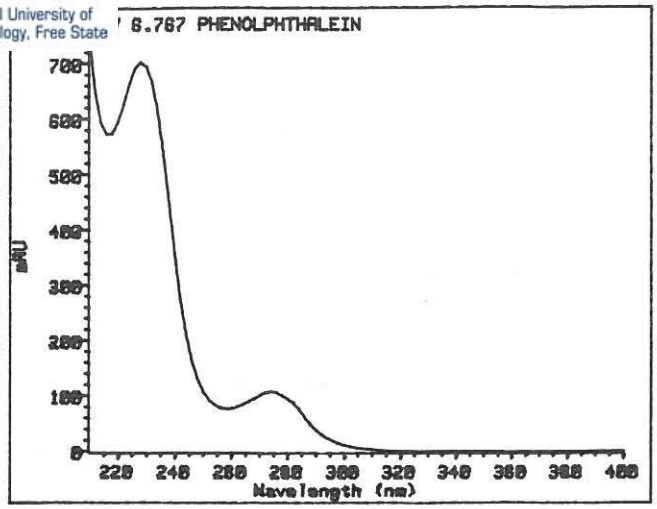


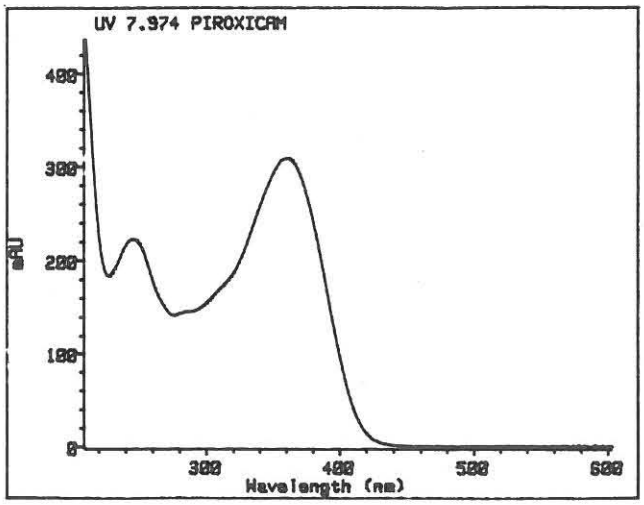


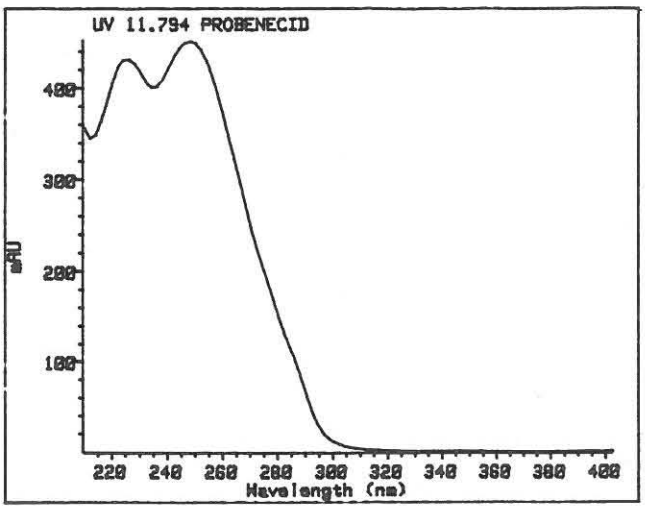


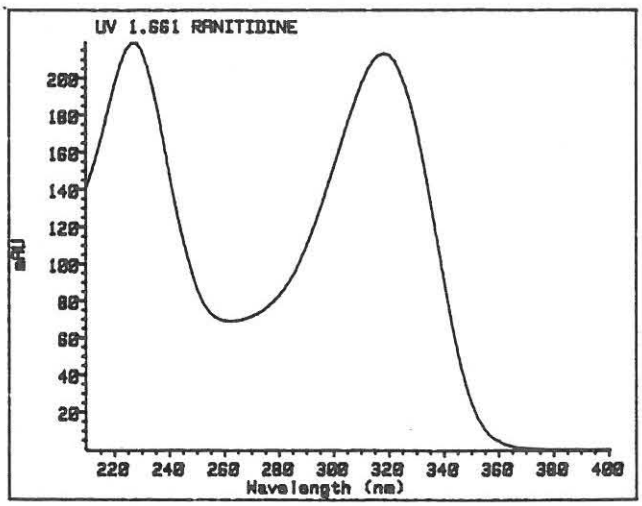


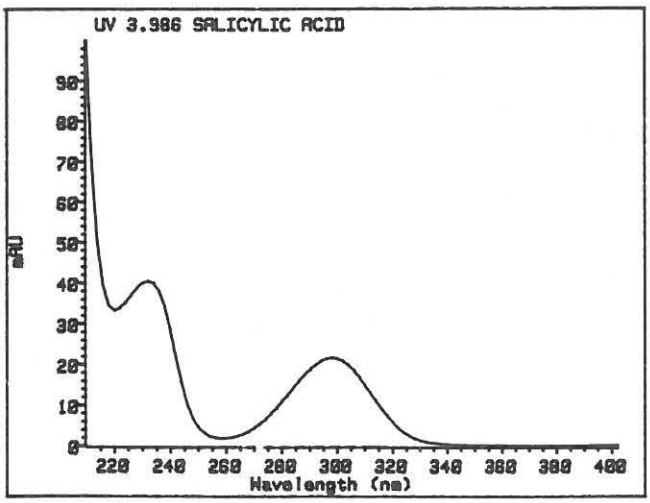


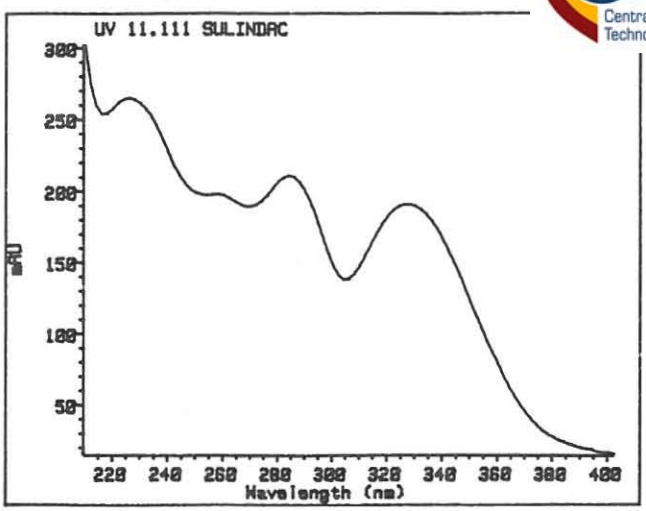


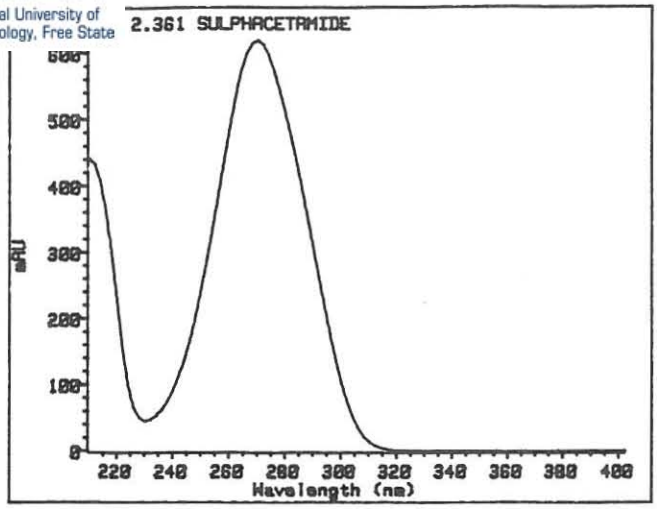


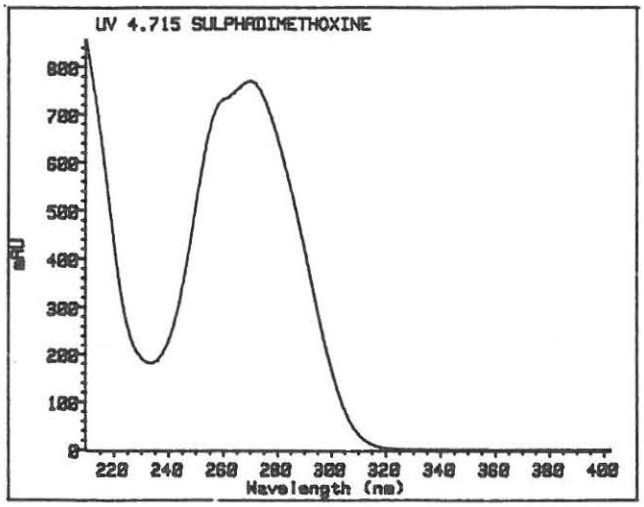


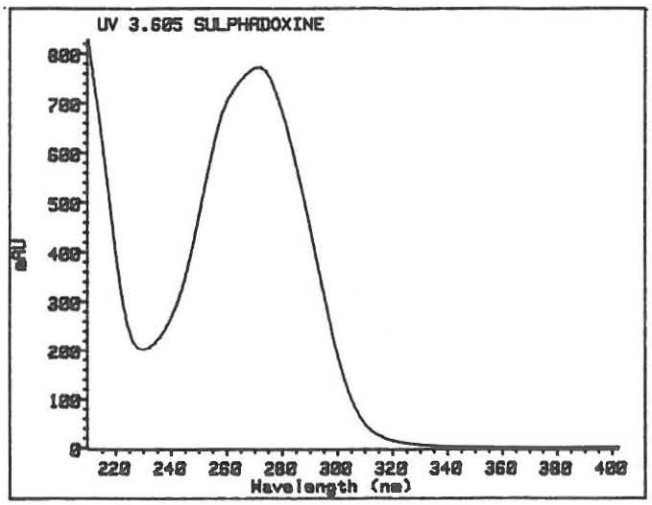


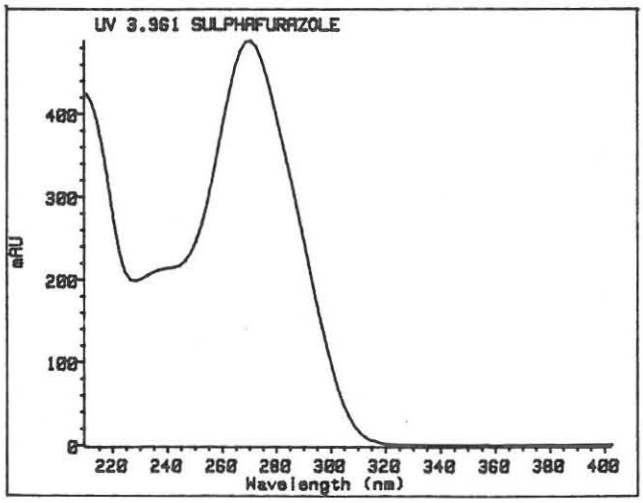


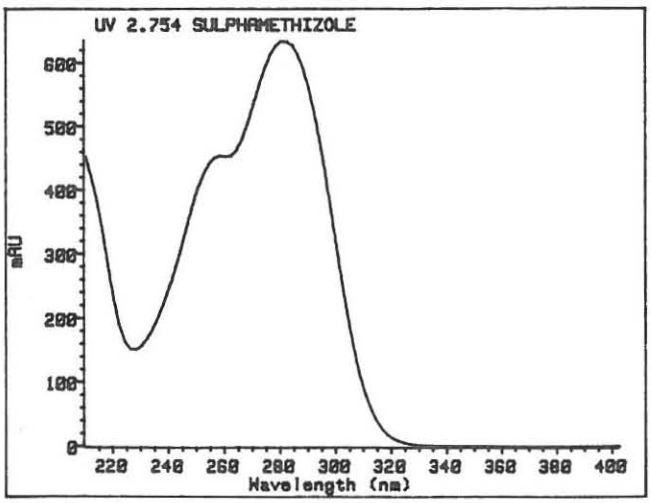


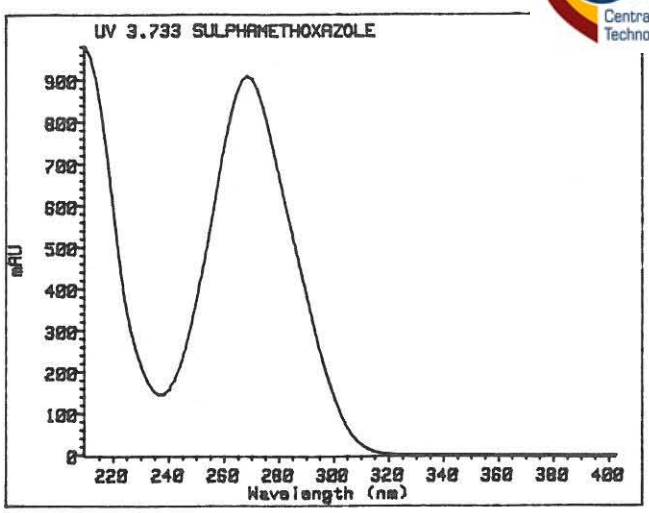


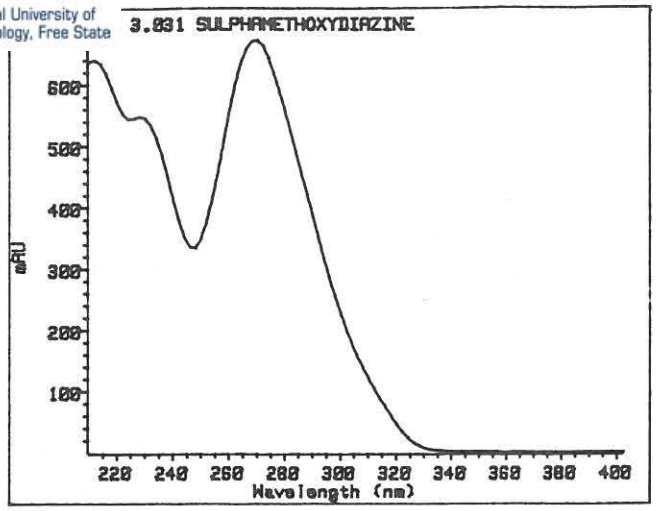


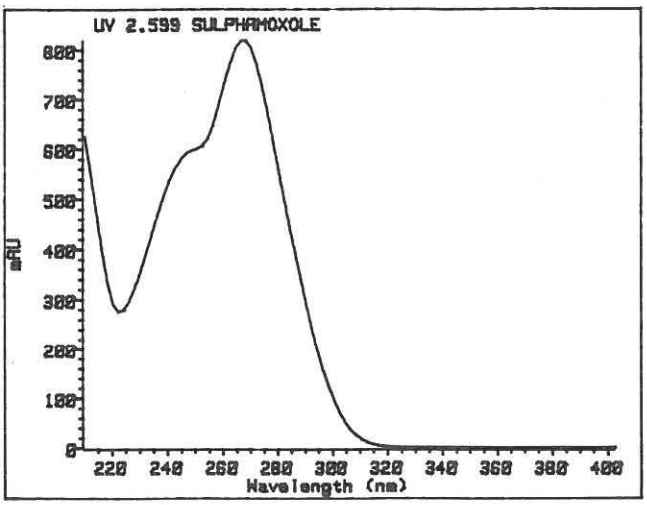


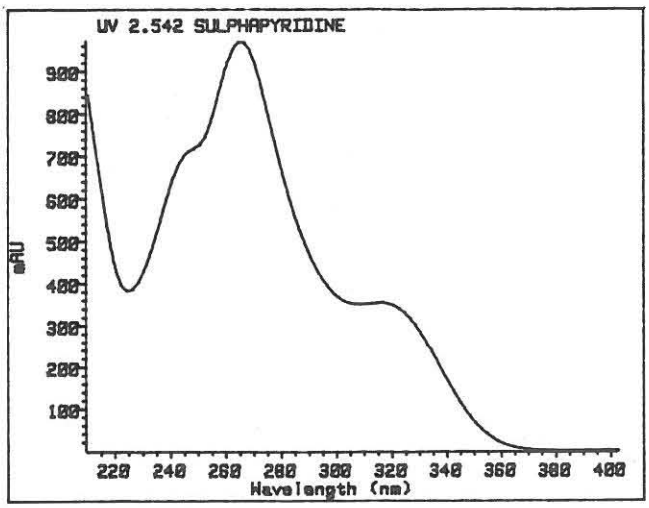


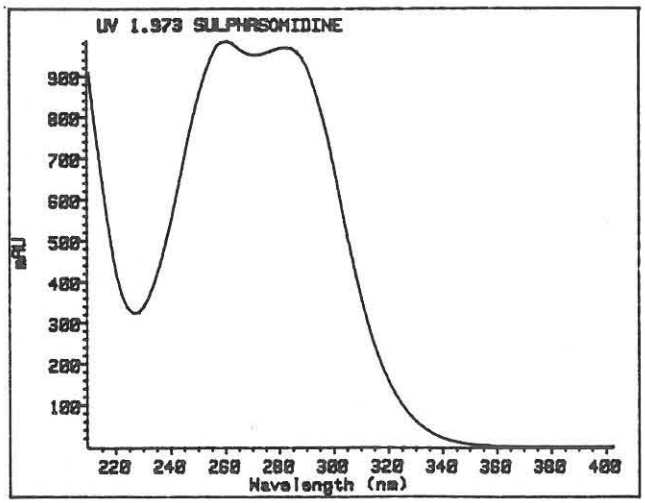


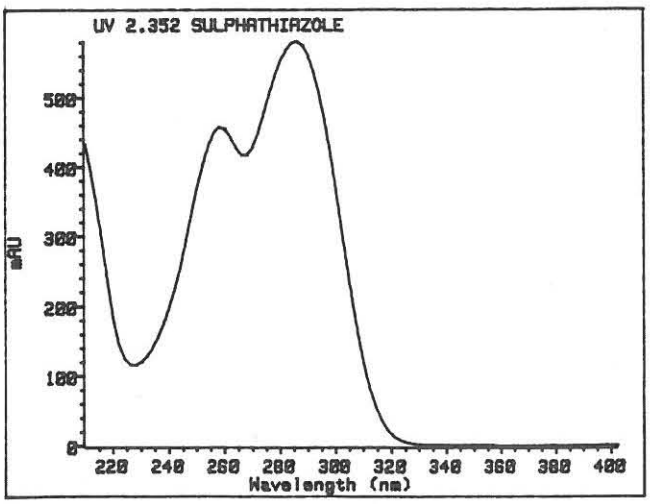


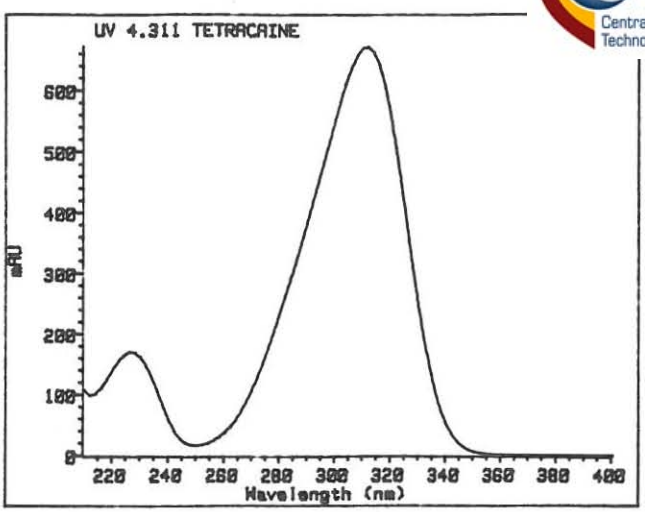


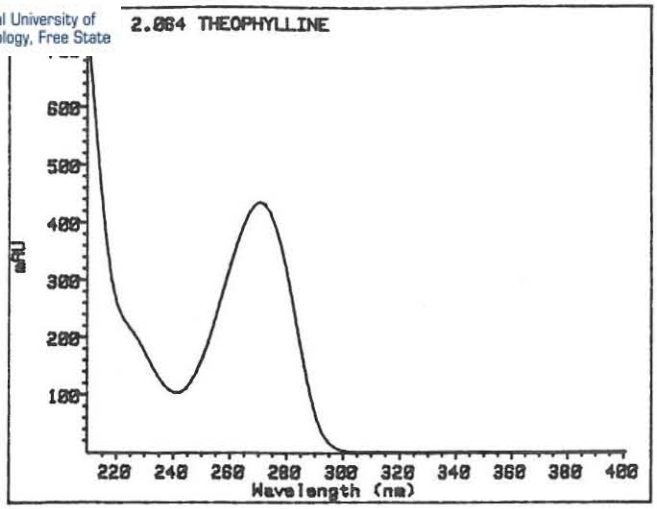


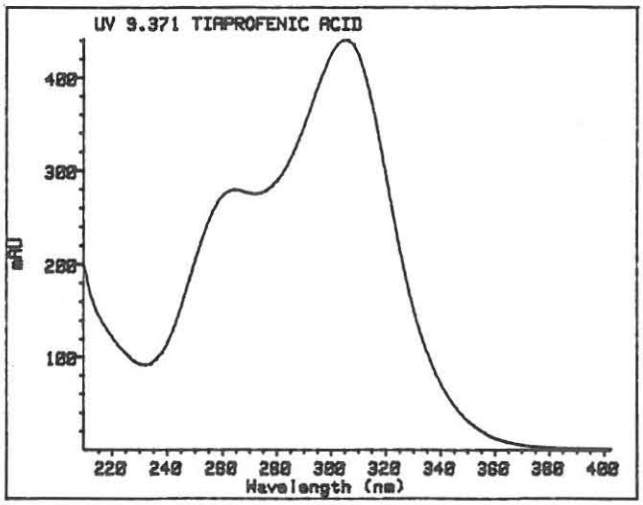


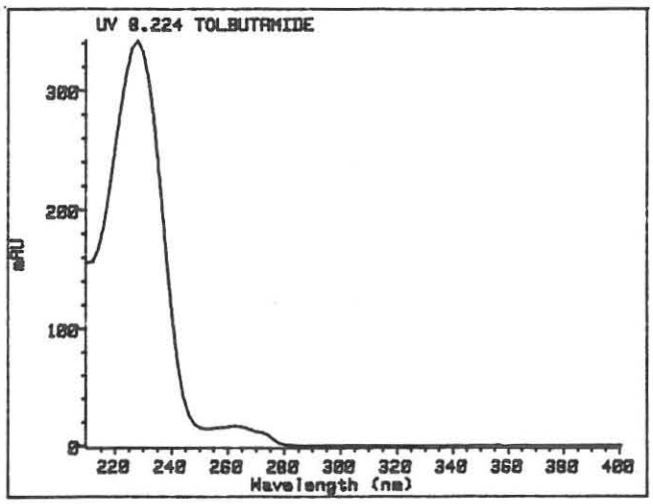


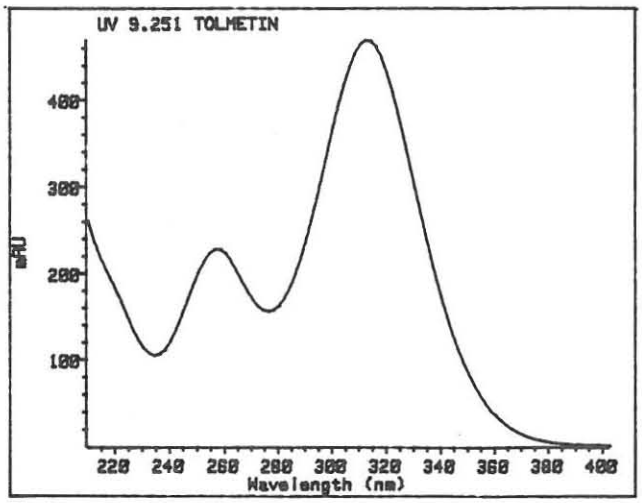


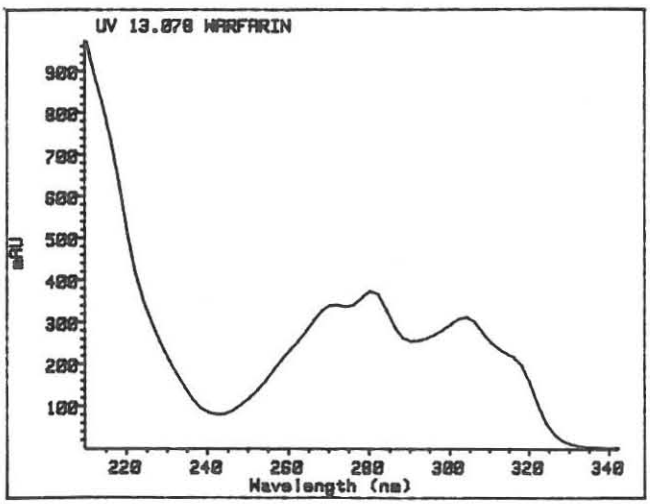












APPENDIX III

ULTRAVIOLET SPECTROPHOTOMETRIC ABSORPTION DATA

(Absorption maxima listed in numerical order of the strongest peak in the spectrum and subsidiary peaks in order of absorbance strength)

λ max (nm)	SUBSIDIARY PEAKS	COMPOUND
218	286/344	Flufenamic acid
218	234/274	Meclofenamic acid
218	278/350	Mefenamic acid
220	262	Ibuprofen
220	266	Mafenide
224	280	Benzthiazide
224	270/316	Hydrochlorothiazide
226		Chlormezanone
226	278	Chlorothiazide
226	248	Probenecid
226	318	Ranitidine
226	284/258/328	Sulindac
228	275	Benzoic acid

228		Bezafibrate
228	300	Glibenclamide
228	274	Phenolphthalein
228	262	Tolbutamide
230	270	Naproxen
232		Chlorpropamide
232		Clopamide
232	274/342	Furosemide
232	298	Salicylic acid
234		Bumadizon
234		Phenformin
236	270	Cyclopenthiazide
240		Acetanilide
246		Acetohexamide
246		Flurbiprofen
246		Paracetamol
256		Ketoprofen
258		Aminophenazone

260	316	Indomethacin
260	282	Sulphasomidine
262		Bisacodyl
264		Acetazolamide
264		Sulphapyridine
268		Sulphamethoxazole
268		Sulphamoxole
270		Sulphacetamide
270		Sulphadimethoxine
270		Sulphafurazole
270	228	Sulphamethoxydiazine
270		Theophylline
272		Caffeine
272		Sulphadoxine
274		Diclofenac
276		Chloramphenicol
276	216	Cinnamic acid
278		Alclofenac

280		Chlorzoxazone
280		Sulphamethizole
280	272/304	Warfarin
284		Carbamazepine
284		Fenbufen
286	334	Niflumic acid
286	258	Sulphathiazole
306	264	Tiaprofenic acid
312	226	Tetracaine
312	258	Tolmetin
362	244	Piroxicam

APPENDIX IV

ULTRAVIOLET SPECTROPHOTOMETRIC ABSORPTION DATA

(Drugs listed in alphabetical order)

COMPOUND	λ max (nm)	Relative Retention time
Acetanilide	240	0.16
Acetazolamide	264	0.09
Acetohexamide	246	0.37
Alclofenac	278	0.39
Aminophenazone	258	0.11
Benzoic acid	228/275	0.17
Benzthiazide	224/280	0.31
Bezafibrate	228	0.48
Bisacodyl	262	0.54
Bumadizon	234	0.65
Caffeine	272	0.12
Carbamazepine	284	0.30
Chloramphenicol	276	0.16
Chlormezanone	226	0.24

Chlorpropamide	232	0.30
Chlorothiazide	226/278	0.10
Chlorzoxazone	280	0.24
Cinnamic acid	216/276	0.24
Clopamide	232	0.19
Cyclopenthiazide	236/270	0.40
Diclofenac	274	0.76
Fenbufen	284	0.51
Flufenamic acid	218/286/344	1.00
Flurbiprofen	246	0.67
Furosemide	232/274/342	0.25
Glibenclamide	228/300	0.86
Hydrochlorothiazide	224/270/316	0.11
Ibuprofen	220/262	0.75
Indomethacin	260/316	0.78
Ketoprofen	256	0.42
Mafenide	220/266	0.06
Meclofenamic acid	218/274/234	1.00

Mefenamic acid	218/278/350	0.95
Naproxen	230/270	0.44
Niflumic acid	286/334	0.76
Paracetamol	246	0.09
Phenformin	234	0.08
Phenolphthalein	228/274	0.30
Piroxicam	244/362	0.35
Probenecid	226/248	0.52
Ranitidine	226/318	0.07
Salicylic acid	232/298	0.17
Sulindac	226/258/284/328	0.48
Sulphacetamide	270	0.10
Sulphadimethoxine	270	0.21
Sulphadoxine	272	0.16
Sulphafurazole	270	0.17
Sulphamethizole	280	0.12
Sulphamethoxazole	268	0.16
Sulphamethoxydiazine	228/270	0.13

Sulphamoxole	268	0.11
Sulphapyridine	264	0.11
Sulphasomidine	260/282	0.09
Sulphathiazole	258/286	0.10
Tetracaine	226/312	0.19
Theophylline	270	0.09
Tiaprofenic acid	264/306	0.40
Tolbutamide	228/262	0.36
Tolmetin	258/312	0.39
Warfarin	272/280/304	0.58

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