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Study on Methods of Simultaneous Multi-Component Analysis.

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Study on Methods of Simultaneous Multi-Component Analysis

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by

Jennifer Bernice Ashie

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ABSTRACT

Study on Methods of Simultaneous Multi-Component Analysis

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Many new instrumentation and different instrumental techniques have been developed to deal with increasing complexity of samples encountered. Many researchers also have coupled these instrumental techniques with chemometric algorithms to assist in the quantitative analysis of multi-component samples in the hope of alleviating the need of tedious separation and cleanup procedures. These newer chemometric procedures tend to be complex and difficult to understand and implement and are successful under different circumstances and conditions. In this study, we start from the very simple beginning and examine the factors that can present difficulties with obtaining the correct results and observe how the system behaves so as to find a better and simpler chemometric procedure to perform mixture quantitative analysis. We have used simulated and actual experimental data obtained from a UV-VIS spectrophotometric measurement of metal complexes to conduct the study. Well understood and defined systems tend to give good results. The main obstacle has been, and still is, interferences in spectral information one gets from the measurement.

DEDICATION

I would like to dedicate this thesis to my parents, Seth A. Ashie and Charlotte T. Ashie, for their support and encouragement throughout my education. Thanks for your loyalty and friendship.

I also dedicate this thesis to my brothers and sister: Joseph, John, Joel, Jeremiah, and Jemima. Thanks to you all and I appreciate you more than I can ever say. May the good Lord bless and keep as all.

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CHAPTER 1

INTRODUCTION

The complexity of analyzing samples with numerous unknown components presents a major challenge in modern instrumental analysis. Most analytes of interest are accompanied by other compounds absorbing in the same spectral region (1), and this leads to the inherent lack of resolution of the classical ultra-violet (UV) spectral measurement (2). In such cases resolution of the components is often associated with cumbersome sample cleanup and separation procedures. However, there are risks associated with separation methods such as loss of analyte, contamination of sample, possibility of incomplete separation, and, above all, the procedure can be expensive and time consuming (1). Separation of the analyte from potential interferences is quite often a vital step in analytical procedures (3). Simultaneous multi-component analysis by UV-visible molecular absorption spectrophotometry are mainly developed for the purpose of minimizing the cumbersome task of separating interferents and to allow determination of an increasing number of analytes, consequently reducing analysis time and cost (4).

Analytical Separation Techniques

There are quite a number of separation techniques that can be employed in the determination of the analytes of interest. The use of traditional methods like extraction is quite difficult because extraction techniques require large solvent consumption with accompanying high cost of disposal. The extraction time is long and generation of dirty extracts requires tedious cleanup steps. Moreover, due to environmental concerns, there has been the need for the development of modern instrumental techniques such as the chromatographic separation methods and spectroscopic methods that are able to perform simultaneous multi-component analysis. The chromatographic separation methods include gas chromatography (GC) (5 - 10), high performance liquid chromatography (HPLC) (11 - 16), and electrophoresis (17 - 22).

Spectroscopic methods include UV-visible absorption (23 - 28), fluorescence spectrophotometry (29 - 34), and mass spectrometry (MS) (35 - 39).

Chromatography

Chromatography separates complex mixtures with great precision. There are quite a number of chromatographic techniques that have been developed to analyze complex mixtures; these include gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE).

Chromatography is a powerful separation method that finds applications in all branches of science. Chromatography encompasses a diverse and important group of methods that allows the separation, identification, and quantitative determination of closely related components of complex mixtures. One of the weaknesses of chromatographic methods is the lack of structural information for the species of interest. It is necessary then to use standards to match retention times (40).

Chromatography is a physical separation method used to analyze complex mixtures. It involves the use of a stationary phase and a mobile phase. In all chromatographic separations the sample is dissolved in a mobile phase (the solvent that is moving through the column), which may be a gas, a liquid, or a supercritical fluid. The stationary phase is fixed in place in a column or on a solid surface, it is most commonly a viscous liquid chemically bonded to the inside of a capillary tube or onto the surface of solid particles packed in the column (41). The parameters used in describing each band of a chromatogram do not express information about the relationships between the bands. Two parameters are used to quantify the amount of mixing of the materials contained in two eluted bands: these are the separation factor and the resolution. The separation factor for two adjacent bands (say 1 and 2) is defined as

$$\alpha_{1,2} = \frac{\mathrm{VR2} - \mathrm{VM}}{\mathrm{VR1} - \mathrm{VM}} = \frac{\mathrm{K2}}{\mathrm{K1}} \qquad [1.1]$$

where; $\alpha_{1,2}$, compares the K_1 and K_2 values of the bands, $V_{R1} - V_M$ is the net retention volume, defined as the difference between the elution volume of peak 1 and the hold-up volume, and $V_{R2} - V_M$ is the net retention volume, defined as the difference between the elution volume of peak 2 and the hold-up volume, K_1 and K_2 represent the capacity factors. The calculation is made with the larger volume (more slowly eluted band) as the numerator. For any pair of bands the resolution is defined by

$$\text{Resolution} = R_s = \frac{\mathrm{VR2} - \mathrm{VR1}}{1/2(w2+w1)} = \frac{\text{peak separation}}{\text{average peak width}} \qquad [1.2]$$

The denominator in this equation is the average of the two baseline widths, and the numerator is the separation of the peaks. The parameter R_s provides a quantitative measure of how much mixing of materials there is between two adjacent bands. The resolution-or degree of separation achieved-is determined by the choice of stationary phase, mobile phase, temperature, and length of the stationary phase through which the separation occurs (41).

Gas Chromatography.

In gas chromatography, the components of a vaporized sample are separated as a consequence of being partitioned between a gaseous mobile phase and a liquid or solid stationary phase held in a column. The gaseous mobile-phase in GC is called the carrier gas and it must be chemically inert. There are two types of gas chromatography; gas liquid chromatography (GLC) and gas solid chromatography (GSC). With GLC, the stationary phase is a nonvolatile liquid bonded to the inside of the column or to a fine solid support, whereas GSC is based on a solid stationary phase in which retention of analytes occurs because of physical adsorption (42).

Gas chromatography (GC) is one of the useful tools available to chemists. It is widely used and capable of separating and analyzing small quantities of sample even of great complexity. Majek et al. (5) described how different multivariate analysis and classification methods can be used to characterize the gas chromatographic separation of complex hydrocarbon mixtures with three columns coupled in series. Three columns with different polarities were used: SE 30, 30 m x 0.32 mm x 0.25 µm (from Machery-Nagel, Germany); SE 54, 25 m x 0.25mm x 0.25 µm(from RIC, Belgium); Nucol (bonded polyethyleneglycol, SUPELCO, Bellefonte, USA), 15 m x 0.25 mm x 0.25 µm (from Supelco, USA). The columns were coupled in series by press-fit connectors. The HP 5890 A (Hewlett-Packard, Avondale, USA) gas chromatograph with split injector and FID was used for all the measurements, the inlet carrier gas pressure was measured by an additional U-manometer with an accuracy of 100 pa. An aneroid manometer was used to measure the outlet pressure with an accuracy of 10 pa. Hydrogen was used as a carrier gas and the oven temperature was 60 ^{o}C. The hydrocarbons used in the model mixture exhibited a slight difference in the chromatographic behavior both on the individual chromatographic columns as well as the column series. This is why multivariate analysis was used to detect these small differences. They observed that in using only the three single columns, the corresponding data matrix gave the same results as with the principal component analysis (PCA). The fact that no additional principal component appeared when the extended matrix was used demonstrated the agreement between observed data and the theoretical assumptions.

A comprehensive two-dimensional gas chromatography (GC x GC) was applied to the quantification of overlapping faecal sterol, this was described in the work done by Truong et al. (6). Standard solutions containing a mixture of seven sterols and 5 alpha-cholestane as internal standard, and sample mixtures that comprised varying ratios of sterol and stanols from green lip mussel tissue and dried cow faeces were analyzed. Quantitative results were compared with single-column GC analysis. It was observed that the single-column GC-flame ionization detection was unable to reliably quantitate target sterols, and the GC x GC experiment permitted small amounts of sterols and stanols to be detected and separated. Separation of 24-ethyl-epi-coprostanol from several algal-derived interfering components was achieved. From their study, they demonstrated that GC x GC technology provided a greater confidence in the quantitative analysis for sterol analysis than for conventional single-column GC. The GC x GC method allows complete separation of peaks of interest which co-elute in normal capillary GC analysis and revealed other peaks in this same region which were obscured in the lower resolution single column technique, demonstrating the enhanced resolving power of the GC x GC system. This results in more reliable and accurate quantification of the components.

A method was developed for the simultaneous determination of trace organic contaminants in seawater and interstitial water samples from Cadiz Bay (SW of Spain). Urban or industrial wastewater discharges and contamination of diverse types from urban and agricultural areas contribute significantly to pollution of the marine environment. As a result, a wide variety of organic contaminants are present in this system including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pesticides. To analyze these semivolatile organic contaminants in marine samples mentioned earlier, the stir bar sorptive extraction technique (SBSE) and thermal desorption coupled to capillary gas chromatography mass spectrometry (SBSE-TD-GC-MS) were used. Seawater samples from different sampling points were collected in bottles of amber-glass (500 mL), filtered (0.45-μm), and placed in a cooler to maintain the temperature at 4 ^{o}C. Interstitial waters were obtained from sediment cores at a sampling point in Cadiz Bay, centrifuged at 4500 rpm for 30 min (5 ^{o}C), and the supertant water was obtained and placed into 20-mL vials. The sample solutions of 100 mL seawater and 10 mL interstitial waters were analyzed. They observed that following the recommended protocols the method was sensitive, robust, and showed a good linearity between 5 and 500 ng L $^{-1}$ for all compounds tested. The method also presented detection limits lower than1 and 10 - ng L $^{-1}$ for 100 mL and 10 mL samples, respectively, and the recovery ranged from 20 to 90% (7).

High Performance Liquid Chromatography.

High performance liquid chromatography is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. Liquid chromatography (LC) has a liquid mobile phase. The great power of liquid chromatography resides in the combination of a wide range of possible mobile-phase properties together with the choice of numerous, significantly different kinds of stationary phases and a wide variety of detectors. Liquid chromatography (LC) is the most widely used of all the analytical separation techniques (43). High performance liquid chromatography (HPLC) is a powerful tool in analytical chemistry. It has been used extensively in chemical analysis (11-14). The reasons for the popularity of this method is its sensitivity, its ready adaptability to accurate quantitative determinations, its ease of automation and its suitability for separating nonvolatile species or thermally fragile ones (11). The components of the HPLC include: a solvent delivery system, a sample injection valve, a high-pressure column, a detector, and a computer to control the system and display results. Columns containing various types of stationary phases are commercially available. Two of the more common stationary phases include normal phase and reverse phase. The phases are selected such that the components of the sample distribute themselves between the mobile and stationary phases to varying degrees. It operates on the same principle as extraction, but one phase is held in place while the other moves past it.

The normal phase operates on the basis of hydrophilicity and lipophilicity by using a polar stationary phase and a less polar mobile phase. Thus hydrophobic compounds elute more quickly than do hydrophilic compounds. The reverse phase operates on the basis of hydrophilicity and lipophilicity (44). The stationary phase consists of silica based packing with n-alkyl chains covalently bound. For example, C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds.

Gennaro, Marengo, Gianotti, and Angioi (12) presented the simultaneous separation of 13 (three mono-, six di-, and four tri-) chloroanilines. They used a conventional reverse-phase HPLC method in which the pH of the mobile phase was controlled. A Merck LiChrospher 100 RP-18 5 µm (250 x 4 mm) endcapped was the stationary phase. The detection was performed at 240 nm where all the species showed significant absorptivity values. In the chromatogram recorded, three well-resolved groups of peaks could be recognized, which corresponds to the mono-, di-, and tri-chloroanilines respectively. This method allows the separation between chloroanilines containing different numbers of chloride (Cl) group, but is not able to separate the isomers. The use of a greater concentration of acetonitrile or of gradient elution could shorten the total analysis time and make closer the retention times of the three groups.

El-Gindy et al. (13) found out that for the determination of two multi-component mixtures containing guaiphenesin, dextromethorphane hydrobrimide, and sodium benzoate together with either phenylephrine hydrochloride, chlorpheniramine maleate, and butylparaben (mixture 1) or ephedrine hydrochloride and diphenhydramine hydrochloride (mixture 2). The HPLC method depended on using an ODS column with mobile phase consisting of acetonitrile - 10 mM potassium dihydrogen phosphate, pH 2.7 (40:60 vol./vol.) containing 5 mM heptane sulfonic acid sodium salt (for mixture 1) and a cyanopropyl column with mobile phase consisting of acetonitrile-12 mM ammonium acetate, pH 5 (40:60 vol./vol.) (for mixture 2) and UV detection at 214 nm. The method was coupled with chemometrics such as principal component regression (PCR) and partial least squares (PLS-1) for the analysis of the two components combinations. The proposed method was simple, sensitive, and less time consuming.

El-Gindy et al. (14) later developed an HPLC method for the determination of two multi-component mixtures containing guaiphenesine (GU) with salbutamol sulfate (SL), methylparaben (MP) and propylparaben (PP), mixture 1; and acephylline piperazine (AC) with bromhexine hydrochloride (BX), methylpraraben (MP), and propylparaben (PP), mixture 2. The HPLC method was developed using a reverse phase (RP) 18 column at an ambient temperature with mobile phase consisting of acetonitrile - 0.05 M potassium dihydrogen phosphate, pH 4.3 (60:40 v/v), with UV detection at 243 nm for mixture 1, and mobile phase consisting of acetoniteile - 0.05 M potassium dihydrogen phosphate, pH 3 (50:50 v/v), with UV detection at 245 nm for mixture 2. Because the simultaneous determination of these compounds in their mixtures is hindered by strong spectral overlap throughout the wavelength range, the HPLC method coupled with partial least squares (PLS-1) and principal component analysis were applied to overcome the problem. The proposed method reduced the duration of the analysis. The methods were validated in terms of accuracy, specificity, precision, and linearity in the range of 20-60 μg mL^{-1} for GU, 1-3 μg mL^{-1} for SL, 20- 80 μg mL^{-1} for AC, 0.2-1.8 μg mL^{-1} for PP and 1-5 μg mL^{-1} BX and MP.

Dudkiewicz-Wilczynska, Tautt, and Roman (15) applied the HPLC methodology to the determination of benzalkonium chloride (BAC) in aerosol preparations. (BAC) is a mixture of alkylbenzyldimethylammonium chlorides. For the HPLC method a column with packing modified with cyano groups and mobile phase containing 0.075 M acetate buffer with acetonitrile (45:55) in isocratic elution was used for qualitative and quantitative determinations and for method validation. The quantitative determination of BAC content in the selected preparations was performed. The determined content corresponded to the declared BAC content in the tested samples. The content was calculated from the sum of areas of the individual BAC homologues peaks present in a given preparation and compared to the sum of the same homologues in the standard. The developed method allowed fast assessment of BAC identity as its homologues migrate between the 14th and 26th minutes. High separability between individual BAC homologues and the other components of the preparations indicated that the method had adequate selectivity and specificity.

Capillary Electrophoresis

Capillary Electrophoresis (CE), which is an instrumental version of electrophoresis, was developed in the mid-to-late 1980s. It has become an important tool for a wide variety of analytical separation problems. It yields high-speed, high-resolution separations on exceptionally small sample volumes (0.1 to 10 nL in contrast to slab electrophoresis, which requires samples in the μL range). The electrophoretic separation technique is based on the principle that under the influence of an applied potential field different species in solution will migrate at different velocities from one another. The movement (migration) of charged specie under the influence of an applied field is characterized by its electrophoresis mobility, $\mu_{e,}$ which has units of $cm^2sec^{-1}V^{-1}$. The velocities of the migrating species depend not only on the electric field but also on the shapes of the species and their environment. The migration rate of an ion (v) depends on the electric field strength. The electric field in turn is proportional to the magnitude of the applied voltage (V) and inversely proportional to the length (L) over which it is applied.

$$v = \mu_e \times \frac{V}{L} \qquad [1.3]$$

The electric field-driven separation can be very rapid and at the same time exhibit excellent resolution making CE a popular technique for analysis (43).

Azhagvuel and Sekar (17) developed a simple, selective, and cost effective capillary zone electrophoresis method for the simultaneous determination of cetirizine dihydrochloride (CTZ), paracetamol (PARA), and phenylpropanlolamine hydrochloride (PPA) in tablets. They found that a 10 mM sodium tetraborate background electrolyte solution (pH 9.0) was suitable for separation of all analytes. An uncoated fused-silica capillary of length 76 cm (effective length 64.5 cm) was used for separation. They reported that all the analytes were completely separated within 10 minutes at the applied voltage of 20 kV, and detection was performed at 195 nm with a UV detector. Ibuprofen was used as internal standard for the quantification of the drugs. Validation of the method was performed in terms of linearity, accuracy, precision, limit of detection, and quantification (LOQ). This method has been applied for the determination of active ingredients in tablets, and the recovery was found to be ≥ 98.60 % with the relative standard deviation (R.S.D.) ≤ 1.56%. The LOQ of the CTZ, PARA, and PPA was found to be 2.0, 2.0, and 4.0 μg mL^{-1}, respectively. There were no interfering peaks due to the excipients present in the pharmaceutical tablets.

Qi et al. (18) simultaneously separated three bioactive triterpenes in Chinese herbs; ursolic acid, oleanolic acid, and 2α, 3β,24-trihydroxy-urs-12-en-28-oic acid by a simple and applicable nonaqueous capillary electrophoresis (NACE) method using methanol: acetonitrile (65:35 v/v) mixtures containing 90 mM trishydroxymethylaminomethane (Tris) at an applied voltage of +25 kV and a hydrodynamic injection of 5 s. They found that electrophoretic medium containing a mixture of solvents was particularly advantageous to achieve high selectivity. It was also found that the analytes were not separated in ammonium acetate and sodium cholate. However, when the Tris was used separation was obtained. This newly established NACE method is suitable for the analysis of the main bioactive triterpenes in Chinese herbs, especially ursolic acid and oleanolic acid.

A selective and rapid capillary zone electrophoresis method for the determination of the multi-component aminoglycoside antibiotic gentamicin is described by Curiel et al. (19). Base line separation of gentamicin C1, C1a, C2, C2a, and C2b components was achieved with a background electrolyte containing 0.35 mM cetyl trimethylammonium bromide, 3% methanol, and 90 mM sodium pyrophosphate (pH 7.4) and detected directly with UV detection without derivatization. Quantitative analysis was performed and it illustrated the potential use of capillary electrophoresis for the identification and quantitation of gentamicin components. However, the application of this method is limited to a gentamicin concentration range of 2-6 mg/mL.

Spectroscopic Methods

In spectrometry, compounds or atoms are identified by their characteristic spectral peaks and their concentrations are determined from the corresponding peak intensities using some kinds of calibration methods. All organic compounds are capable of absorbing electromagnetic radiation because all contain valence electrons that can undergo electronic transitions. Promotion of electrons from low energy ground state orbital to higher energy excited states orbital.

Ultraviolet-Visible (UV-VIS) Spectophotometry

Ultraviolet-visible spectophotometry is defined (45) as a technique usually used to identify substances by analyzing the spectrum produced when the substance absorbs certain wavelengths of ultraviolet and visible light. Spectrophotometric multi-component analysis involves recording and mathematically processing of absorption spectra for samples that consist of several components contributing to the overall spectrum in proportion to their individual absorptivities and concentrations. Ulraviolet-visible spectrophotometry has extensively been used for quantitative determination of components present in a mixture (23-26). This is largely because many molecules absorb radiation strongly in this region. The low cost and the simplicity in operating such instrumentation also add to the advantages of the UV-visible spectrometry. However, spectral interference poses a major limitation when mixture samples are encountered.

The wavelength of UV-visible light absorbed by a molecule depends on the ease of electron promotion. Most applications of absorption spectroscopy of organic compounds are based on transitions from the n to π electrons to the π* excited state because the energies required for these processes bring the absorption bands into the UV-visible region (200 to 700 nm). Both n to π* and π to π* transitions require the presence of unsaturated functional group to provide the π* orbital. Molecules containing such functional groups are capable of absorbing UV-visible radiation are called chromophores (3).

In principle the analyte concentration is linearly related to absorbance as given by the Beer Lambert Law.

$$A = -\mathrm{Log}\ T = \mathrm{Log}\ \frac{Po}{p} = \epsilon\ b\ c \qquad [1.4]$$

where: A is absorbance and its unit is dimensionless, concentration c has units of moles per liter (M), path length, b, in centimeters (cm), and molar absorptivity, ϵ, in (mol L^{-1} cm^{-1}) (46).

Quantitative spectrophotometry has been greatly improved by the use of a variety of multivariate statistical methods, particularly principal component regression (PCR) and partial least squares regression (PLS).

Simultaneous determination of dexamethasone and two excipients (creatinine and propylparaben) in injections were presented by Collado et al. (23). They applied the UV-spectroscopy with a multivariate calibration method. For the quantitative determination of the analyte of interest, a training set of 15 samples with a central composite design was prepared for calibration, with the concentration of dexamethasone lying in the known linear absorbance-concentration range. These samples were prepared by dilution of a convenient amount of stock solutions. The resolution of the three-component mixture in a matrix of excipients was accomplished by using partial least squares (PLS-1). Notwithstanding the elevated degree of spectral overlap, they have been able to rapidly and simultaneously determine the amount of the analyte with high accuracy and precision with no interference. In the calibration step a simple and fast method for wavelength selection was used.

A method for the simultaneous spectrophotometric determination of the divalent ions of iron, cobalt, nickel, and copper based on the formation of their complexes with 1, 5-bis(di-2-pyridylmethylene), thiocarbonohydrazide (DPTH) was proposed by Garcia Rodriguez et al. (24). Samples were prepared in 25-mL standard flasks by taking the required volume of the solution to be analyzed to obtain Co, Ni, Fe, and Cu concentrations over their respective linear determination ranges, with the final solution containing a total metal concentration lower than 3 µg mL^{-1}. PCR, PLS-1, and PLS-2 methods were used to analyze the spectra of the samples under study and to calculate the concentration of Co (II), Ni (II), Fe (II), and Cu(II) in the mixture. From the results it was observed that best recovery values were obtained by PLS-2 method for absorbance data. The satisfactory results indicate that the method would be effective for the analysis of samples of similar complexity.

The Davidon-Fletcher-Powell (DFP) iterative algorithm was used (25) for the simultaneous quantitation of the components of complex mixtures from their UV-vis spectra. First, the effect of noise, overlap between standard spectra, and the starting point for resolving numerically generated spectra was investigated. The average concentrations calculated were highly accurate and their real values were all within the confidence interval for the calculated concentrations. Then, spectra for the pure solutions of the mixture components were used. The method was applied to the resolution of active principles in various pharmaceutical preparations. The results were correct because the algorithm assigned the most significant concentration values to those components actually present in the sample and comparatively very low or even zero values to those absent from it. Also, the calculated concentrations were very close to their real counterparts for all the samples.

Neves et al. (26) described in their work the development and evaluation of the method of simultaneous determination for iron(III) and copper(II). The simultaneous spectrophotometric determination of copper and iron was based on the yellow and red azide complexes formed in 50% (v/v) water/acetone medium. All the reagents used were chemically pure. Sodium azide was purified by dissolution in water, filtered, and precipitated with pure ethanol. The precipitate was dried under vacuum and then at 110^{o}C. A 3.0 M standard sodium azide solution was prepared. Standard copper(II) solution (0.010 M) was prepared by dissolving $CuSO_4.5H_2O$ in distilled water containing 0.001 M perchloric acid, and Standard iron(III) solution (0.010 M) was prepared by dissolving Fe (NH_4) $(SO_4)_2.12H_2O$ in 0.01 M perchloric acid solution. The solutions were standardized by EDTA titrations. Working standard solutions were prepared as needed by suitable dilutions. Absorbances of the test solutions were measured at 345 and 435 nm against a blank of (0.1 M $HClO_4$), where the molar absorptivities for the iron(III) complexes were 8.77×10^3 and 8.49×10^3 $Lmol^{-1}cm^{-1}$, respectively, and molar absorptivities for copper(II) complexes were 1.47×10^3 and 5.69×10^3 $Lmol^{-1}cm^{-1}$, respectively. The metal ion concentrations were calculated by using the simultaneous linear equation. The relative standard deviations were 0.86% for iron(III) and 1.6% for copper(II). The better precision for iron(III) was as a result of the fact that the wavelengths used correspond to maxima in the spectra for iron(III) complexes, while the 345 nm value is on the descending portion of the spectrum for the copper(II) system.

Fluorescence

Fluorescence is a reliable and accurate means for detecting and quantifying compounds. It is a phenomenon in which light energy is absorbed by a molecule and then re-emitted again as a photon of light with a longer wavelength. The usefulness of fluorescence methods is being increasingly recognized for their excellent sensitivity, selectivity, non-invasiveness, and speed.

The phenomenon is common among organic molecules including groups of strongly fluorescent dyes such as fluorescein (absorbs blue, emits yellow-green light), the rhodamines (absorb green light, emit orange-red), and the family of stilbene optical brighteners that absorb UV-VIS light and emit blue light. Fluorescence is measured by means of a fluorometer. It measures the amount of fluorescence produced by a sample exposed to a given monochromatic radiation. The application of fluorescence in research has necessitated the design of various instruments for the measurement of fluorescence at the least possible cost without compromising accuracy, precision, sensitivity, and selectivity (47).

Fluorescence as a means for multi-component quantitative analysis has also been actively pursued (29-34). With different instrumentation and chemical manipulations, a number of workers (30-32) have achieved good quantitative results for a variety of challenging multi-component systems.

Sikorska et al. (30) demonstrated in their work an application of front-face fluorescence spectroscopy combined with multivariate regression methods to the analysis of fluorescent beer components. Fresh and illuminated beers were used for the assays of riboflavin and aromatic amino acids. The samples were degassed in an ultrasonic bath before measurements were taken to avoid light scattering by the CO_2 bubbles. Partial least-squares regression (PLS-1, PLS-2, and N-way PLS) were used to develop calibration models between synchronous fluorescence spectra and excitation-emission matrices of beers, on one hand, and analytical concentrations of riboflavin and aromatic amino acids, on the other hand. The best results were obtained in the analysis of excitation-emission matrices using the N-way PLS2 method.

Ho et al. (31) applied the method of rank annihilation for quantitative analysis of multi-component fluorescence data that were acquired in the form of an excitation-emission matrix (EEM) by the video fluorometer. A scattered light EEM for the pure solvent was similarly acquired. This scattered light EEM was subtracted from each EEM prior to mathematical analysis. In this work innovative instrumentation and novel mathematical algorithms were combined. With their set of data, they amply demonstrated that the method of rank annihilation is a powerful tool for quantitative multi-component analysis.

In 1987, Nithipatikom and McGowan (32) described determination of multi-component systems using phase-resolved fluorescence spectroscopy (PRFS) and synchronous excitation to combine the dimensions of fluorescence lifetime and emission and excitation wavelength for five-and six-component systems of spectrally overlapping polycyclic aromatic hydrocarbons. Experimental conditions such as the wavelength intervals scanned, the difference maintained between the emission and excitation monochromators, and the wavelength and detector phase angles used to generate the data matrixes were all generalized rather than optimized for the particular component used in the study. The accuracy was better for the PRFS determinations than for steady-state synchronous excitation determinations using the same number of equations in the data matrix. An average relative error of 0.34% was found for the PRFS determinations of the five-component system, compared with-1.5% obtained for the steady-state determinations with the use of 24 equations. An average relative error of 3.8% was obtained for the PRFS determinations of the six-component system, also with the use of 24 equations. The selectivity derived from the fluorescence lifetime dimension in PRFS was therefore shown to be important in multi-component determinations using generalized conditions for data acquisition and proved valuable of samples that require both qualitative and quantitative analysis.

Mass Spectrometry

Mass spectrometry is a powerful tool that provides a positive identification of a compound with a high degree of specificity. Mass spectrometers are often coupled with gas or high performance liquid chromatographic systems or capillary electrophoresis columns to permit the separation and determination of the components of complex mixtures. The process involves separation of species of ions by mass from each other by fragmentation of a molecule and transmission of these ions to the mass spectrometer for analysis. The mass spectrometer consists of an ion source, a mass analyzer, transducer, and a recorder that are operated under high vacuum conditions. The accelerated ions pass from the source into a number of types of analyzers. The ions are separated according to mass to charge ratio and the heavy and the lighter ions are deflected, whereas the ions with the appropriate mass to charge ratio pass through to the detector, then the signal is picked up by the recorder. Mass spectrometry (MS) allows identification of molecular and atomic species. However, a major difficulty arises when the different species present in a mixture of components are introduced simultaneously into the source (48).

Some research had been done using the centrifugal microparticulate bed chromatographic technique combined with mass spectroscopy to separate and identify components in a mixture without the necessity of isolation and purification after chromatography. Karasek and Rasmussen (35) reported that no difficulty was encountered in running the mass spectra in the presence of SiO_2. The presence of too much H_2O caused the separating efficiency of the centri-chromatograph to fall off drastically, hence its presence should be minimized for that reason. Experimental data presented for a mixture of anthracene and N-mehtyl-2, 3-diphenylindole showed good separation between the components.

The use of direct sampling mass spectrometry coupled with multivariate chemometric analytical techniques was explored for the analysis of sample mixtures containing analytes with similar mass spectra by Gardner et al. (36). Water samples containing varying mixtures of toluene, ethyl benzene, and cumene were analyzed by purge-and-trap/direct sampling mass spectrometry. The multivariate quantitation methods were found to be superior to univariate regression when a unique ion for quantitation could not be found.

Durant, Dumont, and Narine (37) developed a simple method for the determination of free fatty acids, phytosterols, mono, and diglycerides present in canola oil deodorizer distillate (DD) and soapstock samples. Canola oil produced in Canada is the world's third leading source of vegetable oil. It is obtained from the seeds of *Brassica napus* and *Brassica rapa* containing low erucic acid and flucosinolates cultivars. The analytes were derivatized "in situ" using a mixture of hexamethyldisilazane (HMDS), pyridine, and trifluoroacetic acid, then separated by gas chromatography (GC) with mass specteometry (MS) for final detection. The chromatographic conditions used in their work allowed for the separation and quantification of oleic, linoleic and linolenic acids, mono olein, and monolinolein in both samples and brassicasterol and α-tocopherol in deodorizer distillate samples. Mass spectrometry provided an accurate identification for the compounds that were at very low concentrations (> 0.09%).

Oleic acid was the most abundant compound in both samples. The compounds were identified by comparing with the standards.

Simultaneous Multi-Component Analysis

Simultaneous multi-component analysis by absorption measurements based upon ultraviolet and visible radiation is one of the most extensively used tools by analysts for quantitative and qualitative analysis. This process avoids the prior separation procedures involving, extraction, concentration of constituents, and the cleanup steps that make the process time consuming. Simultaneous multi-component analysis by absorption measurements is one of the most sensitive measuring techniques and is fast and simple but lacks the inherent selectivity to allow direct application to highly complex materials that analyst are faced with in modern times because the absorption spectra overlap severely. Thus, simultaneous multi-component analysis by absorption measurements based upon ultraviolet and visible radiation is often coupled with chemometrics to help with the quantitation of the unresolved peaks (49).

Advantages of Simultaneous Multi-Component Analysis

Simultaneous multi-component analysis avoids the separation techniques that might be required, hence samples remain intact because the various species are determined simultaneously in a mixture. With the simultaneous multi-component analysis by absorption measurements, spectral data are readily acquired with ease, the process is fast, accurate, and simple. Other important characteristics of simultaneous multi-component analysis using the spectrophotometric method includes; 1. wide applicability to both organic and inorganic systems, 2. typical detection limits of 10^{-4} to 10^{-5} M, and 3. moderate to high selectivity.(50)

Difficulties of Simultaneous Multi-Component Analysis

Considering the advantages associated with simultaneous multi-component analysis by absorption measurements, one may assume that the method is perfect. However, there are some shortcomings associated with this method. Although the spectral data are readily acquired, they are usually broad, featureless, and overlap severely. This makes it difficult to quantitate the components present because the peaks are unresolved and it requires longer interpretation times due to significant data interpretation challenges. A common problem is the choice of complexing agents. Spectrophotometric reagents may be rather unselective, that is quite a number of metals form complexes with very similar absorption spectra, and these systems are not appropriate for selective determination of metal ions by multi-component analysis. On the other hand, there are complex forming agents such as 1, 10-phenanthroline that form complexes with several metal ions, but only a few of them highly absorb in the visible spectral range. This again limits the number of simultaneously determined metal species to say two or three that might even have quite similar absorption spectra. (51). In order for the purpose of quantitative simultaneous multi-component analysis of components present in unresolved spectra to be achievable, in this work the use of mixed organic reagents was proposed. This method is often coupled with chemometic methods to enable correct interpretation of results.

CHAPTER 2

METHODS OF SIMULTANEOUS ANALYSIS OF COMPLEX MIXTURES

The complexity of modern samples and the need for quantitative analysis of the constituents in these complex mixtures has prompted many workers to develop new instrumentation capable of quickly acquiring data from which the identities and concentration of the components can be readily determined. Separation techniques are commonly used to assist in analysis. The simultaneous determination of individual components present in a mixture solution has been performed using instrumental approaches. These procedures avoid the difficult task of separating interferents and allow determination of an increasing number of analytes, consequently reducing analysis time and cost. In principle sprctrophotometric analysis of several components simultaneously is based on measurements of absorbances at a number of selected wavelengths of at least as many as the number of components to be determined according to Beer's Law. Quantitative spectrophotometric analysis of mixture components is featured for systems with low spectral selectivity, namely in the ultraviolet, visible, and infrared spectral range (52).

Chemometrics

Chemometrics has extensively been used in analytical chemistry. It is defined in the chemical discipline as the use of mathematical and statistical methods to analyze chemical data to provide maximum relevant information. It enables analysts to correctly interpret results. Since the 1980s, rapid developments in computer science, microelectronics, and chemometrics have spurred greater advances of simultaneous multi-component analysis (53). Multivariate calibration techniques including regression and factor analysis have been widely used

Regression Analysis

Regression analysis is a statistical technique used for the modeling and analysis of numerical data consisting of values of a dependent variable (response variable) and one or more independent variables (explanatory variables). In regression, an equation is developed for the purpose of prediction. When a prediction is made about the value of a single dependent variable Y from one independent variable X, the relationship between them is assumed to be linear the equation is of the form

$$\check{y}_x = C + MX \qquad [2.1]$$

where $\check{y}_x$ is the predicted value of Y corresponding to X, M is the slope of the regression line, and C is the y-intercept of the regression line. This is called simple linear regression.

When more than one independent variable is incorporated into the prediction of the dependent variable Y and the relationship between each independent variable and Y is linear, then this process is called multilinear regression, and the regression equation is of the form

$$Y' = b_1X_1 + b_2X_2 + . . . + b_kX_k + b_o \qquad [2.2]$$

where b_1, b_2 . . . $b_{k,}$ are the coefficients of the independent variables X_1, X_2, $X_{k,}$ and b_o is the constant term.

In general, the dependent variable in the regression equation is modeled as a function of the independent variables, corresponding parameters ($\beta_{o\,,}$ β_n) ,and an error term ε. The error term is treated as a random variable. It represents unexplained variations in the dependent variable. The model that describes the relationship with an error has this form:

$$Y_i = \beta_o + \beta_1X_i + \varepsilon_i \qquad [2.3]$$

where Y_i is the ith observation of the dependent variable, X_i is the ith observation of the independent variable, β_o and β_1 are the parameters of the model, and ε_i is the random error of $Y_{i\,.}$ The parameters are estimated to give a 'best fit' of the data. Most commonly the best fit is evaluated by using the least squares method (54).

The Method of Least-Squares

The method of least-squares is perhaps the most frequently used method of estimating the concentrations of several components in a mixture sample. The calculation of concentrations of n components in spectrophotometric analysis has been generally regarded as a process of solving a set of n simultaneous linear equations (obtained by selecting absorbancies at n wavelengths) in the n unknowns (concentrations). As n becomes large, this method exhibits great sensitivity to small errors in the experimental data. This method will yield the best estimates in terms of smallest squared errors of the analyte concentrations that the calibration spectra for the entire sample components are included in the analysis (52). For a two-component mixture with a known concentration (X_1, X_2) of the standards, the absorbances of the calibrating solutions for each standard (X_1, X_2) are measured and recorded within a wavelength range. Matching cuvettes are often used with path length (b) of 1 cm. The molar absorptivity (ε) of each component (X_1 and X_2) is obtained from each wavelength by applying Beer Lambert's law:

$$Y_{Total} = \varepsilon_{1,\lambda i}\, b\, [X_1]_{,\lambda i} + \varepsilon_{2,\lambda i}\, b\, [X_2]_{,\lambda i} \qquad [2.4]$$

where : Y_{Total} is the total absorbance of the mixture, $\varepsilon_{1,\lambda i}$, $\varepsilon_{2,\lambda i}$, are the molar absorptivities of component 1 and component 2 at the ith wavelength, respectively. However, the concentration of $[X_1]$ and $[X_2]$ in the mixture is not known. To find $[X_1]$ and $[X_2]$ the absorbance of the mixture can be calculated by guessing the concentrations. The guesses do not have to be close to correct values. Both guesses are arbitrarily chosen. The calculated absorbance is defined as:

$$A_{calc} = \varepsilon_{X1}\, b[X_1]_{guess} + \varepsilon_{X2}\, b[X_2]_{guess} \qquad [2.5]$$

The least-squares condition is to minimize the sum of squares $(A_{calc} - A_m)^2$ by varying the concentrations $[X_1]_{guess}$ and $[X_2]_{guess}$. EXCEL has a powerful tool called solver that can be used to carry out the minimization (55). This procedure is readily extended to mixtures containing more than two components. Absorbance measurements at more wavelengths than there are components in the mixture gives good and accurate results.

The least-squares methods seek a minimum value for an error matrix equal to the difference between the measured and a calculated matrix. This method is the same as the conventional solution of the matrix equation with the exception that the minimum is found for a matrix instead of for a vector (56). Generally, analytical procedures require proper calibration if they are to provide reliable results. Considerable amount of research done with the method of least-squares have been published (34, 57, 58, 59, 60). The method of least-squares yields predictably reliable results only if one has knowledge of all the major constituents present. Among the methods summarized by Warner et al. (34) and Sternberg et al. (57) the least-squares fitting techniques can be strongly affected by not accounting for all the sample constituents that might be present.

Warner et al. (34) described how quantitative information can be obtained from fluorescent mixtures using the method of least squares or linear programming, based on previously determined calibrated excitation-emission matrices (EEM) of known components, even in cases of severe overlap and poor signal/noise ratio. Three sets of experiments were run corresponding to mixtures of one-, two-, and three-component systems. The first experiment was designed to show the linearity of the fluorescence intensity. Five solutions of free base octaethylprorphin (H_2OEP) were prepared at various concentrations. The correlation coefficient of calculated concentration vs. volumetrically determined concentration was 0.9997. Their results indicate that the fluorescence is linear and reabsorption and quenching processes are negligible. The second system analyzed was a mixture of free base octaethylporphin (H_2OEP) and free base tetraphenylporphin (H_2TPP). There was a significant overlap between these two components in both excitation and emission. Hence, the least squares algorithm was applied to the system. The least squares fitting the standard matrices to the mixture samples gave satisfactory results. In comparing the known concentrations of H_2OEP and H_2TPP showed that the errors were 0.8% and 7%, respectively. Finally, they analyzed a three-component system of zinc octaethylporphin (ZnOEP), tin (IV) dichlorooctaethylporphin ($SnCl_2OEP$), and H_2OEP. The matrices were analyzed over the wavelength ranges of 450 to 646 nm in excitation and 550 to 746 nm in emission. The data were obtained in 4-nm increments, producing a 50 x 50 martix of 2500 data points. The least squares algorithm was applied. The calculated concentrations for ZnOEP and H_2OEP were satisfactory, but the $SnCl_2OEP$ was 13% in error. This 13% error in the three-component system described above could be attributed to the overlap between ZnOEP and $SnCl_2OEP$ because these components differ by only 3 or 4 nm, indicating a significant overlap.

Sternberg et al. (57) also established that the method can be developed and be applicable for the analysis of other complex mixtures especially those involving components with other highly overlapping spectra. Commercial grades of ergosterol obtained from Parke Davis and Co. and Nutritional Biochemical Corp. were employed in the irradiation work without further purification. Three of the components of the irradiation mixture were used in the preparation of the synthetic mixtures: ergosterol, lumisterol, and calciferol. Isopropyl alcohol was used as the solvent throughout the work. Solutions of known compositions consisting of ergosterol, lumisterol, and calciferol in varying proportions were prepared from the pure components. The ultraviolet absorption spectra of the synthetic mixtures and the pure components were determined. Plots of absorbancy at various wavelengths against concentrations were obtained from the ultraviolet absorption spectra of the irradiated solutions. A linear relationship was found between absorbancy and over-all concentratin of the irradiated mixture. The standard deviation from linearity was found to be only±0.012 absorbancy units.

The spectra of the synthetic mixtures of known composition were compared with absorbancies calculated from the spectra of the individual components and the composition of the solution to establish the additivity of absorbancies of the pure components. This comparison was made at intervals of 5 mμ in the wavelength range 230 to 300 mμ and a standard deviation (δ) was calculated for each synthetic mixture. The data verified the additivity of absorbancies of components in a mixture within the limits of experimental error. The system studied was modified and a least-square matrix method was employed as an analytical curve fitting technique to provide analysis of the complex ergosterol irradiation mixtures using the ultraviolet spectrophotmetric data.

The least-square methods allow the rapid analysis of binary pharmaceutical formulations with minimum error. Mahalanabis et al. (58) described the least-square method in the matrix form for the simultaneous determination of rifampicin and isoniazed in a mixture. They used the K-matrix representation of Beer's law, which constituted a least-square method in the matrix form. For the determination of the standard mixture (K'), 20 mg of standard samples of rifampicin and isoniazid were accurately weighed and each dissolved in 50 mL of solvent, (methanol-water 70 : 30). Enough solvent was added to make the volume up to 100 mL. Then 2 mL of this solution was diluted to 50 mL with solvent and the absorbance of the solution was taken from 230 nm to 290 nm at 5-nm intervals versus the solvent. The determination of rifampicin and isoniazid content in capsules was carried out.

The individual ultraviolet spectra of rifampicin and isoniazid in methanol-water (70: 30) show substantial absorbance over the wavelength range 230-290 nm. The least-squares method appears to be valid in the working range of 230-290 nm for standard solutions containing up to 19.55 µg mL^{-1} of rifampicin and 10.1 µg mL^{-1} of isoniazid. The limit of detection was 3.84 and 2.32 µg mL^{-1} of rifampicin and isoniazid, respectively. These results indicate the high degree of accuracy of the proposed least-squares method.

Erdal Dinc (59) developed the multivariate spectral calibration methods. These are the Tri-linear regression-calibration (TLRC) and Multi-linear regression-calibration (MLRC) for the multiresolution of a ternary mixture of caffeine (CAF), paracetamol (APAP), and metamizol (MET) whose spectra closely overlap. Twenty tablets of CAF, APAP, and MET were accurately weighed and powdered in a mortar. An amount equivalent to one tablet was dissolved in 0.1 M HCl in a 100-mL calibrated flak and the solution was filtered into a 100- mL calibrated flask through Whatman number 42 filter paper. The residue was washed three times with 0.1 M HCl. The individual spectra of CAF (λ max = 272.6 nm), APAP (λ max = 242.7 nm), MET (λ max = 258.4 nm), and their mixture spectrum were observed in the spectral region 220-320 nm. The calibration algorithms TLRC and MLRC were applied to the multiresolution of the three-component mixture CAF-APAP-MET system. As an alternative, the classical least-square method (CLS) was used to solve the problem. For this purpose, standard series of solutions of CAF, APAP, and MET in 0.1 M HCl were prepared. Their absorption spectra were recorded over the wavelength range 220-320 nm against a blanc (0.1 M HCl). The absorptivity a value of the three compounds CAF, APAP, and MET was calculated using the absorbancies measured at nine selected wavelengths. Using the absorptivities value, a system of equations with nine unknowns was written for the compounds in the ternary mixture. The matrix was solved and the concentration of CAF, APAP, and MET in the mixture were determined.

Goicoechea and Olivieri (60) reported the use of multivariate spectrophotometric calibration for the analysis of two decongestable tablets, where paracetamol is the principal component and diphenhydramine or phejylpropanolamine are the minor components. For the analysis of the active components in the decongestable tablets Benadryl Day and Night, 20 tablets of each pharmaceutical were ground and mixed. The amounts corresponding to the equivalent of one tablet was dissolved, in each case, in 1000 mL of doubly distilled water. The solutions were stirred for 15 minutes, filtered, and diluted. The contents of paracetamol-diphenydramine and paracetamol-phenylpropanolamine were simultaneously determined using electric absorption measurements together with PLS-1 multivariate calibration analysis. However, the related multivariate method, classical least-squares method (CLS) has been shown to be unreliable in quantitating the studied components in the mixture.

If some of the constituents are not known, other methods such as non-negative least squares (61) and factor analysis (62-68) have been suggested as possible algorithms.

Factor Analysis

Factor analysis is a statistical approach that can be used to analyze interrelationships among a large number of variables and to explain these variables in terms of their common underlying dimensionality (factor). The statistical approach involves finding a way of condensing the information contained in a number of original variables into a smaller set of dimensions (factors) with a minimum loss of information. Generally, the number of factors is considerably smaller than the number of measures and, consequently, the factors tersely represents a set of measures (69).

Ritter et al. (62) in their work, “Factor Analysis of the Mass Spectra of Mixture”, showed that factor analysis method can accurately determine the number of components in a series of mixtures. They prepared mixtures from high purity samples of a number of materials and these were purposely chosen from representative compounds with mass spectra that were similar to enable them to test the method on the most demanding types of mixtures. Four sets of mixtures, known and unknown were examined. These were: 1. cyclohexane/cyclohexene; 2. Hexane/cyclohexane; 3. Heptane/octane; 4. Unknown xylenes. They worked on each set with great detail to fully clarify the procedure. Four mixtures of cyclohexane and cyclohexene were the source of mass spectra. These mixtures contained, respectively, 80%, 60%, 40%, and 20% by volume cyclohexane. The mass spectra of these mixtures, including those peaks that were used in the analysis, were obtained. Twenty m/e positions were used and, after normalization of the resulting mass spectral system matrix, followed by premultiplication of the normalized matrix by its transpose gave the covariance matrix, then the covariance approximations were used. The results obtained from the approximations showed there were zero residuals; hence the mixture contained two components. The digitized data used for the other three sets of mixtures were obtained and the factor analysis method was applied. They observed that in every case the factor analysis method correctly determined the number of components in the mixture. Impressive results were obtained for the two cases where initial analysis seemed to be in error (the cyclohexane/cyclohexene and heptane/octane mixtures). They pointed out that the method is computationally simple, rapid, and easy to implement on a laboratory minicomputer.

Principal Component Analysis (PCA)

PCA is a useful chemometric technique for finding the underlying repeating patterns in data of high dimensions. Its earliest and most extensive applications were in the psychological and social sciences (70). It has more recently been applied to a wide variety of chemistry problems. PCA is used to identify new and meaningful underlying variables and expresses data in such a way as to highlight the similarities and differences. It is also used to reduce the dimensionality of the data by performing a covariance analysis between factors. Another advantage of PCA is that once a pattern is found in a data set the data can be compressed, i.e. by reducing the number of dimensions without loss of information. PCA makes no assumption about curve shape, the number of components, or their spectra. It provides a relatively rapid way of determining how many components are present.

Considering a two-dimensional data with variables A and B, to find out if A and B variables are related in any way, PCA can be used. It involves the identification of patterns. The PCA (71) selects a new set of axis for the data; they are selected in decreasing order of variance within the data. The first principal component (PC) axis is the line that goes through the centriod but also minimizes the square of the distance of each point to that line. Thus, in some sense, the line is as close to all of the data points as possible. Equivalently, the line goes through the maximum variation in the data points. The second PC axis must go through the centroid and also go through the maximum variation in the data points but with a certain constraint: it must be completely uncorrelated (i.e. at right angles or "orthogonal") to PC axis-1 (72)

The theory and application of PCA in spectrometry have been discussed by several workers (53, 70, 71).

Davis et al. (63) applied the method of principal component analysis to a two-component system. In their work they used real and simulated data, obtained by means of a PDP-11/20 computer that employed 1-8 user BASIC modified for on-line real-time control and data acquisition. Because they considered only binary mixtures, only two eigenvectors were retained. The scalars were calculated and plotted on suitable scales for both axes so as to "fill" the page of plotting paper. In the simulations of mass spectrometry, a chromatographic peak of Gaussian shape and standard deviation (δ) were generated for each species. Each peak was 20-30 points wide (4 δ) out of a total of 200 points. Then the corresponding mass spectrum was formed using the relative weights created for each mass channel. The final spectrum was a channel-by-channel sum of the values of the pair of contributing species. Noise was added to the simulated data from a random-number generator that had a probability distribution of uniform amplitude. The real mass spectrometric measurements were performed using two different systems. For carbon dioxide, masses 44 and 45 were measured and for the mixtures of n-hexane and n-heptane, masses 42, 43, 56, 57, 70, and 71 were selected because they were presented in the spectra of both hydrocarbons.

The results they obtained for the simulated data showed that the extent of peak separation had an effect on both the percentage of the trace of the matrix accounted for by the first vector and on the shape of the resulting scalar plot. For the real data, the separation of the isotopic carbon species of carbon dioxide was a trivial example for two reasons. First, the electron-multiplier signals for masses 44 and 45 were clearly independent of one another. Second, because only two masses were measured, there could be no reduction in the number of vectors necessary to reproduce the original data. The hexane-heptane case was a more realistic one. The second component was easily detected; however, they observed that the percentage of the trace represented by the first vector was high, and it did not change much. The fact that it did decrease at intermediate values of (δ) appears to be real, and it was been attributed to tailing of the peaks.

Yanwei et al. (64) proposed a new normalization method based on PCA. They obtained methanol and chloroform and stored them over 4A molecular sieves before using. Behenic acid without further purification was also used. A solution of 0.5 mg/mL was prepared dissolving it in the 1:1 (v/v) mixture of methanol and chloroform. The spectra were obtained at room temperature. Each spectrum was normalized with the first principal component of PCA, which can represent the main information of the spectrum. From their observation, the PCA normalization method works very well with not only two overlapped peaks but also with three and four overlapped peaks. To demonstrate the success of their data pretreatment, they simulated the Gaussian-type peaks and the combination of Lorentzian-type and Gaussian-type peaks. They observed that all the simulated works showed that the proposed PCA normalization method could correct the misleading synchronous spectra significantly.

Rank Annihilation Factor Analysis (RAFA)

Rank Annihilation Factor Analysis (RAFA) is a method of calculating the concentration of a given component in the presence of other, possibly unknown, two-dimensional data matrices. It was first proposed by Ho et al. (31) in analysis of fluorescence data in the form of an emission excitation matrix (EMM). The RAFA method takes advantage of the unique property of these images, that of producing a matrix with rank equal to unity. The pure image of the quantified components is multiplied by a scalar value and the product is subtracted from the mixture's image. When the scalar is equal to the ratio "amount in sample/ amount in standard", the resultant matrix will have a rank lower by one than the rank of the mixture's data matrix (31). It was modified by Lorber to yield a direct solution of a standard eigen-value problem (56).

Several methods have been proposed for quantifying individual components in mixture, e.g. in overlap chromatographic peaks. The least squares method suffers from the condition that prior knowledge of all components is needed. RAFA is more general in scope. The RAFA method has been successfully tested in excitation/emission fluorescence (31, 33).

The method of rank annihilation qualitatively has been described by Ho et al. (31). They reported that for a multi-component solution, emission-excitation matrix (EEM); M, the rank, ideally, should equal the number of components. If one of the components present in the solution is known say N, and if the correct amount of N is subtracted from M, the original rank of M is reduced by one. It was observed in that case that eigenvalues of (M) corresponding to N becomes zero. The eigenvalues cannot be expected to vanish completely because of errors in the actual experimental data. However, it would attain a minimum. The amount of N subtracted to achieve a minimum in the corresponding eigenvalue would correspond to the relative concentration of the known component in the mixture.

The method of rank annihilation has been shown to be useful for analysis of multi-component fluorescence data acquired by the video fluorometer in the form of an excitation-mission matrix (EEM) (32, 65). With this method, concentrations of known components were computed independently from the EEM of a sample whose complete qualitative composition was known. Ho, Christian, and Davidson applied the simultaneous rank annihilation to a six-component polynuclear aromatic hydrocarbons by using data acquired by the video fluorometer in the form of excitation-emission matrix. This method, called simultaneous multi-component rank annihilation (SMRA), was efficient and yielded satisfactory results (65).

Raoof, Amir, and Bahram (66) proposed a spectrophotometric method for the simultaneous determination of iron, aluminum, and vanadium in the presence of Triton X-100 as neutral micellar media. This method is based on the reaction between analytes and morin at pH 4.0. To each series of 10 mL volumetric flasks, 2.5 mL of solution buffer, 2.0 mL of Triton X-100 (5 %) and 1.5 mL of morin stock solution were added and diluted to the volume with bidistilled water. The solutions were allowed to remain in a Thermostat at 25 (±) ^{o}C and then 3.0 mL of these solutions were transferred into the quartz cell of the spectrophotometer. The solution was titrated with metal ions solutions by means of a micro-syringe in 2.5 minute intervals. After the addition of each aliquot of the metal ions, the spectra of the solutions were recorded in wavelength range of 300 to 500 nm. Due to the high spectral overlapping observed between the absorption spectra for their components, PLS-1 multivariate calibration approaches were applied. The rank annihilation factor analysis (RAFA) of the complexation data suggests that morin forms adduct with the metal ions in a single step.

Niazi et al (67) used RAFA to the spectrophotometric determination of acidity constant of three popular indicators as methyl orange, methyl red, and methyl violet in pure water,

water-TX-100, water-SDS, and water-CTAB micellar media solutions at 25 °C and ionic strength of 0.1 M after each pH adjustment the solution is transferred into the cuvette and the absorption spectra of methyl orange, methyl red, and methyl violet in pure water at various pH values were recorded. Results show that the acidity constant of these indicators are influenced as the percentage of neutral, cationic, and anionic surfactants added to the solution. Also RAFA is an efficient chemometric algorithm for complete analysis of acid-base equilibrium systems by spectrometric method.

Hemmateenejad et al. (68) proposed a two-rank annihilation factor analysis (TRAFA) method for the determination of the acidity constants of diprotic acids. To evaluate the performance of their proposed method, it was firstly applied to simulated data with different spectral characteristics of protogenic species (i.e. H_2A, HA^-, and A^{2-}). The simulated absorbance spectra were calculated according to normal Gaussian distribution between 300 to 600 nm with an interval of 1 nm. Three sets of experimental data were used. Calmagite was used first as a reference compound with known acidity constant. And the method was applied to the determination of the acidity constants of two new chromenone derivatives: BH1 as a mono-protic and BH2 as a diprotic acid. Analysis of a large number of simulated data sets with different relative successive dissociation constants and varying spectral overlapping between the protogenic species was carried out. The proposed TRAFA method was able to determine the acidity constants of diprotic acids even for systems with overlapping spectra. In addition, the calculated pKa2 and pKa3 for calmagite by TRAFA (i.e., 7.95 and 12.05, respectively) were close to the literature values (i.e., 8.1 and 12.4, respectively). Moreover, the acid dissociation constants of the two newly synthesized chromonone derivatives (i.e., BH1 and BH2) in different binary mixed solvents of methanol and water were determined by the proposed method. It was found that the acidity constants were increased by increasing the methanol contents of the binary solvents.

Research Objective

Based on the discussions in Chapters 1 and 2, it has become important for analytical purposes to establish new methods capable of analyzing a large number of samples in a short period with accuracy. Spectroscopic techniques can generate a large amount of data within a short period of time. When coupled with chemometric tools, the quality of the spectral information can be markedly increased, making this combined technique into a powerful and highly convenient analytical tool (31). This has prompted many workers to develop new instrumentation capable of quickly acquiring data from which the identities and the concentrations of the components can be readily extracted.

The method of least-square regression is one of the most widely used methods in estimating the concentrations of several components in a mixture sample and this method yields reliable results only if one has knowledge of all the major constituents present. Warner et al. (34) applied the method of least-squares to quantitatively obtain information from fluorescent mixtures based on previously determined calibrated excitation-emission matrix (EEM) of known components. The least-square fitting technique was satisfactory for the analysis of a three-component mixture. One major disadvantage is the accuracy, which depends on how fast the instrument scans.

The method of rank annihilation is capable of quantifying a particular component known to be present in the mixture without having also to know the identity of the rest of the components. Ho et al. (31) used the method of rank annihilation for the quantitative analysis of a multi-component fluorescent mixture, using the excitation-emission matrix (EEM) acquired by the video fluoremeter. This method gave reliable results for the determination of one component in a mixture even when the identity of the other components is unknown. However, rank annihilation requires the use of statistical programs to quantitatively analyze the data, and this can be time consuming. On the other hand, both methods gave accurate results and that indicates chemometrics is an accepted statistical technique used today.

With the above discussion, a research project with the following objective is proposed;

1. To use the UV-Visible spectrophotometric technique to obtain mixture data.
2. To compare the known simplest and the more sophisticated chemometric methods in quantitative analysis of multi-component data.
3. To know and understand their capabilities and shortcomings in analysis of mixture data with different degree of overlap.
4. To apply the findings on experimental mixture data obtained.

CHAPTER 3

EXPERIMENTAL METHODS

Real Spectra of Fe(III) and Cu(II) Azide Complexes

Instrumentation

A Shimadzu model UV-1700 double-beam spectrophotometer, manufactured by Shimadzu Corporation Analytical Instruments Division (Kyoto, Japan), with a fixed slit width of 1 nm equipped with UV- Probe 2.21 software was used for all absorbance measurements. The system includes a 20-W halogen lamp and a silicon photodiode detector. All absorption spectra were recorded using quartz cells of 1.00 cm path length.

Reagents Used

All chemicals used were of American Chemical Society (ACS) analytical-reagent grade and deionized water was used for preparation of solutions throughout the experiment. Sodium azide,iron(III) nitrate nonahydrate, coppe(II) nitrate, acetone, HNO_3, and $HClO_4$, all ACS reagent grade were all from Fisher Scientific (Fair Lawn, NJ) were used to prepare solutions. Deionized water used was obtained from US Filter Company (Pittsburgh, PA).

Preparation of Standard Solutions

Sodium Azide Standard Solution

Sodium azide standard solution (3.00 M) was prepared by dissolving 19.5 g of sodium azide in 50 mL of deionized water. The solution was transferred to a 100-mL volumetric flask, diluted to the mark, and stored in a glass container when not in use.

Iron(III) Standard Solution

Iron(III) standard solution (0.010 M) was prepared by dissolving 0.404 g of $Fe\ (NO_3)_3$. $9H_20$ in 50 mL deionized water containing 0.010 M HNO_3. The solution was transferred to a 100-mL volumetric flask, diluted to the mark, and stored in a glass container when not in use.

Copper(II) Standard Solution

Copper(II) standard solution (0.010 M) was prepared by dissolving 0.250 g of Cu $(NO_3)_2.2\frac{1}{2}\ H_2O$ in 50 mL deionized water containing 0.0010 M $HClO_4$. The solution was transferred to a 100-mL volumetric flask, diluted to the mark, and stored in a glass container when not in use.

Preparation of Individual Working Solutions

Individual working solutions of iron(III) and copper(II) azide complexes were prepared for spectral measurements. Iron(III) working solution was prepared by pipetting 1.00 mL of iron(III), 2.5 mL of standard sodium azide solution, and 12.5 mL of acetone into a 25-mL volumetric flask and the volume adjusted with deionized water. Copper(II) working solution was prepared by pipetting 0.5 mL of copper(II), 2.5 mL of standard sodium azide solution, and 12.5 mL of acetone into a 25-mL volumetric flask and the volume adjusted with deionized water.

Preparation of Calibration Standard Solution

In preparing the iron(III) azide calibration standard solutions, 1.0 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL of the iron(III) azide working solution were pipetted into five different 5-mL volumetric flasks and then diluted to the mark with deionized water. In preparing the copper(II) azide calibration standard solutions, 1.0 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL of the copper(II) azide working solution were pipetted into five different 5-mL volumetric flasks and then diluted to the mark with deionized water. The absorbances of these standard solutions were then measured on the Shimadzu spectrophotometer.

Preparation of Mixtures

Mixtures of iron(III) and copper(II) azide complex solutions were prepared by pipeting aliquots of the two working solutions with different ratios of the two ions. For the 1:1 ratio solution, 1.0 mL of iron(III) azide complex and 1.0 mL of copper(II) azide complex working solutions were pipetted into the same 5-mL volumetric flask, and the volume was adjusted with deionized water. A 2:1 ratio solution was prepared by pipetting 2.0 mL of iron(III) azide complex and 1.0 mL of copper(II) azide complex working solutions into the same 5-mL volumetric flask and the volume was adjusted with deionized water. Then for the 3:1 ratio solution, 3.0 mL of iron(III) azide complex and 1.0 mL of copper(II) azide complex working solutions were pipetted into the same 5-mL volumetric flask and the volume was adjusted to the mark with deionized water.

Experimental Procedure

The absorption spectra of the working solutions of iron(III) and copper(II) azide complexes and that of the mixture solutions prepared as described above were acquired using the Shimadzu UV-visible spectrophotometer,within a wavelength range of 325-500 nm. The measurements were done against deionized water blank. A pipette was used in transferring aliquots of each standard solution into the cuvettes used. For the iron(III) azide complex calibration standards, prepared, the absorption spectra were obtained individually. Similarly, for the copper(II) azide complex calibration standards, the individual absorption spectra were obtained.

Absorbance data were also obtained for mixtures of iron(III) and copper(II) azide complexes. The mixtures with volume ratio of iron(III) to copper(II) of 1:1, 2:1, and 3:1 were made as described and the absorbances measured from 325-500 nm.

From the absorption spectra obtained for the various mixtures of iron(III) and copper(II) azide complex solutions, judicious selection of wavelengths was made to generate the calibration curves. The wavelengths selected were at an absorbance maximum or the wavelength where the absorbance of only one of the two metal complexes was large or where minimal interference occurs. This is done to achieve better accuracy and precision.

The absorbances of the standard solutions of iron(III) and copper(II) azide complexes, with known concentrations of the analytes are plotted against concentrations at the selected wavelengths. A straight line graph was obtained for all the plots. Based on the equation of the straight line, the molar absorptivities were determined, which equal the slope of the line. The spectral absorbances measured from the unknowns were arranged as sets of two equations and two unknown from which the concentration of the two metal ions in the unknown mixtures was calculated.

The concentrations of the various solutions of iron(III) and copper(II) azide complex standard solutions used in generating the calibration curves are shown in Table 1.

Table 1. Concentration data for calibration: volumes of Fe(III) and Cu(II) azide working solutions pipetted and diluted in a 5-mL volumetric flask, and the concentrations calculated.

Solution (mL)	Concentration (M)	
	Fe(III)	Cu(II)
1	8.0 x 10 $^{-5}$	4.0 X 10 $^{-5}$
2	1.6 x 10 $^{-4}$	8.0 X 10 $^{-5}$
3	2.4 X 10 $^{-4}$	1.2 X 10 $^{-4}$
4	3.2 X 10 $^{-4}$	1.6 X 10 $^{-4}$
5	4.0 X 10 $^{-4}$	2.0 X 10 $^{-4}$

The concentrations of the iron(III) and copper(II) present in the mixture were calculated using simultaneous equations. Then the method of least-squares was also applied to the experimental mixture data to quantitatively determine the concentration of each metal ion present in the unknown mixtures. For the two-component mixtures with known concentrations of the individual standards, the absorbance data of each iron(III) and copper(II) azide complex standards were taken within a wavelength range. This method should yield the best estimates in terms of smallest squared errors of the calculated analyte concentrations versus the expected values because calibration spectra for the entire sample components were included in the analysis.

All the data obtained from these experiments were analyzed using the EXCEL programs; EXCEL solver and EXCEL matrix operation, in Microsoft Office suites 2007 (Microsoft Corporation Redmond Washington). Examples of the method of simultaneous linear equation and the method of least squares using EXCEL are shown in Appendix A, Table 1 and Appendix B, Table 1.

Simulated Spectra

Simulated spectral data were also generated to carry out the proposed studies. Normal distribution was used in generating the spectra. After all the spectral parameters were generated, the first sets of base spectra generated were component (I) and component (II) with featureless spectra. Simulated two-component mixtures of these two base spectra were then generated with varying degree of overlap. The spectra of the two components were normalized to 100%. Figure 1 shows the spectra of component (I) and component (II), while Figure 2 is the spectrum resulting by mixing component (I) and component (II) at 1:1 ratio. Other two-component mixtures were made by keeping the relative simulated concentration of one component such as component (II) constant at 1, while the relative concentrations of the other component, component (I) are varied from 0.25, 0.5, 1.0, 2.0, and 4.0 multiples of the base concentration. Similarly, a second set of mixtures was made, this time keeping component (I) relative concentration constant at 1, while the relative concentration of component (II) was varied from 0.25, 0.5, 1.0, 2.0, and 4.0 multiples of the base concentration. The second set of simulated two-component mixtures of these two base spectra were generated with a varying degree of overlap. The spectra of the two components were again normalized to 100% as before. The method of solving two sets of linear equations with two unknowns and the method of least-squares were both applied to these data to determine the relative concentration of each component in the mixtures. Then two-component mixtures were made by keeping the relative simulated concentration of one component constant at 1, while the relative concentrations of the other component are varied from 0.25, 0.5, 1.0, 2.0 and 4.0

multiples of the base concentration and vice versa as was done previously. The degree of overlap was varied from slight to high.

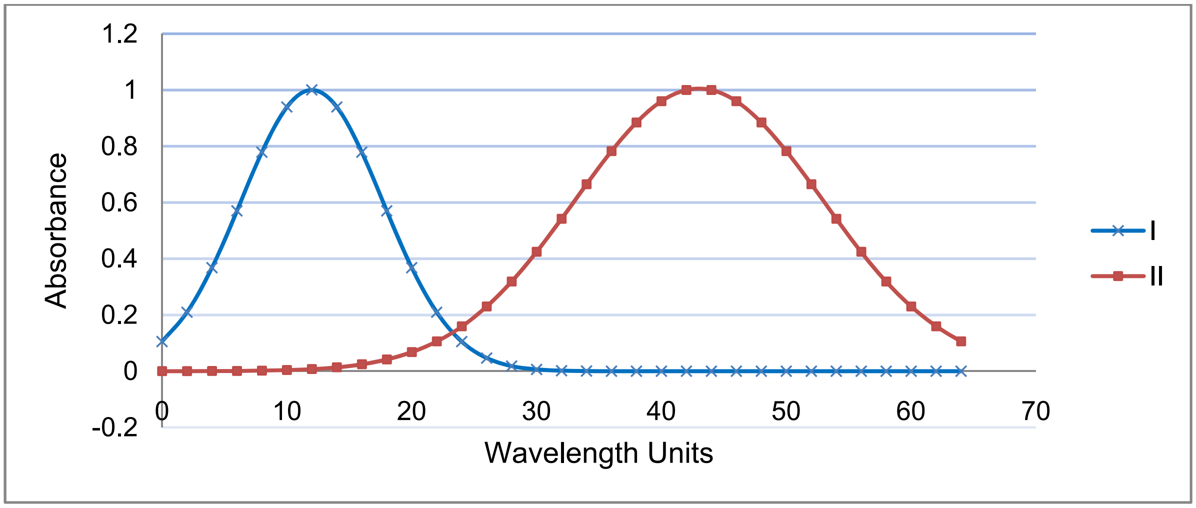

Figure 1. Spectra of component (I) and component (II): Spectra of the two-components at 1:1 ratio, normalized to 100%.

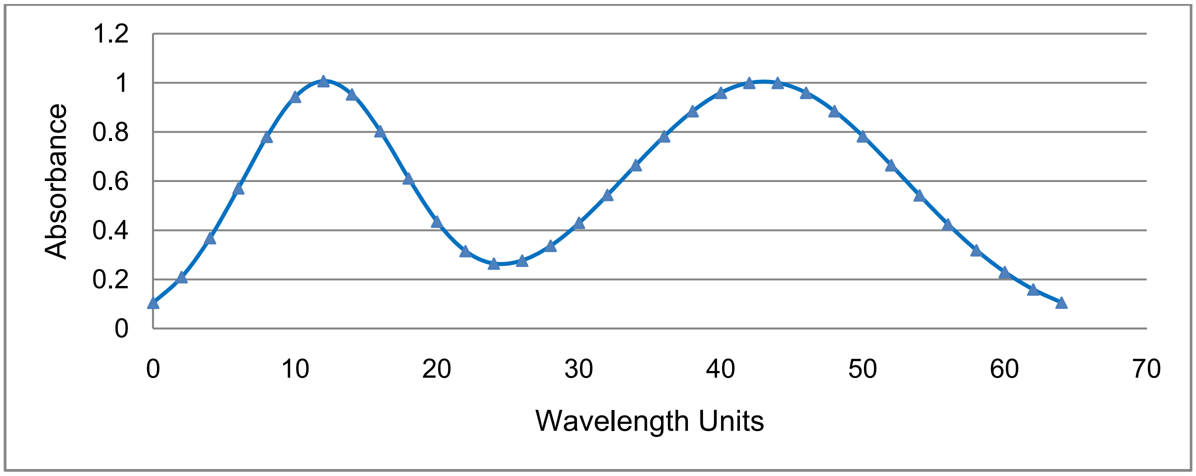

Figure 2. Spectrum of the two-component mixtures (broad spectra): Component (I) and component (II) at 1:1 ratio of base concentration.

In this manner two sets of mixtures in terms of degree of overlap were generated. The first set consists of two sets of mixtures with varying relative concentrations with a minimal spectral overlap and the second set, similarly, consists of two sets of mixtures with varying relative concentrations with a severe spectral overlap.

Another set of base spectra was generated that has components with high spectral feature. Simulated two-component mixtures of these two base spectra were generated again as the previous set of generated spectra, varying the degree of overlap and at similar relative concentration. These components with greater spectral features are shown in Figure 3 and the 1:1 mixture spectrum of the two components is shown in Figure 4.

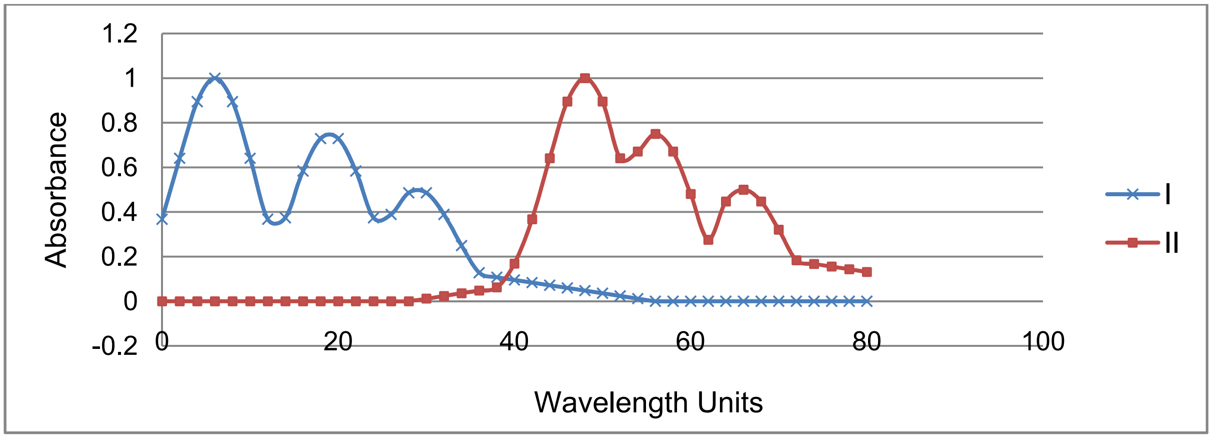

Figure 3. Highly featured spectra: Component (I) and component (II) at 1:1 ratio, normalized to 100 %.

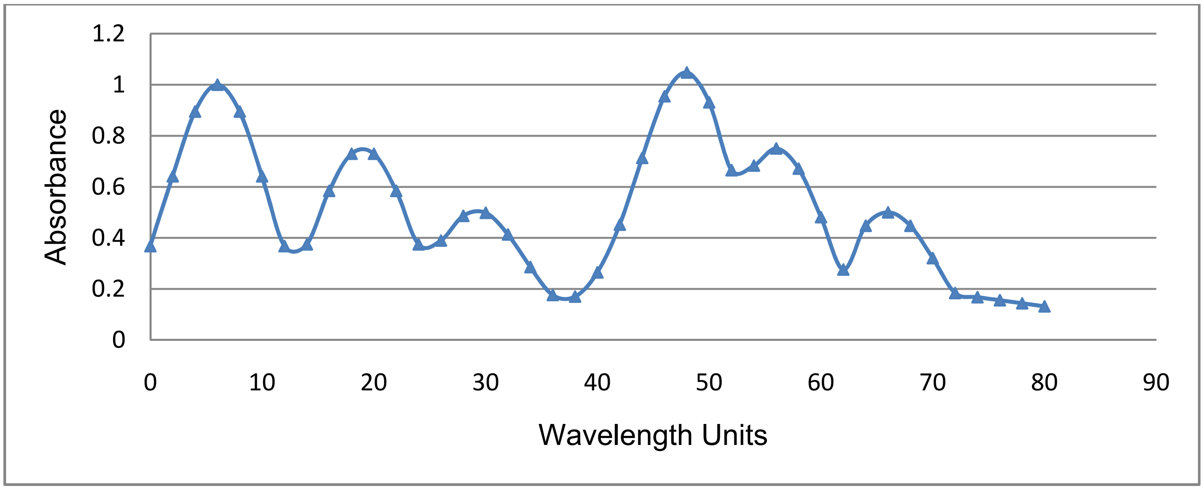

Figure 4. Spectrum of the two-component mixtures (featured spectra): Component (I) and component (II) at 1:1 ratio of the base concentration.

Another method that was also introduced to aid with the determination of relative concentration of each component in the mixture is the total spectral subtraction method which is similar to the rank annihilation method.

Method of Total Spectral Subtraction

In the total spectral subtraction procedure, spectral data of the component whose concentration is to be determined is subtracted from the spectral data of the mixture. The subtraction proceeds with an initial guess of its relative concentration in the mixture. The base spectrum of the analyte component is multiplied by this guessed value. The residual spectrum after subtraction is squared and the sum of the squares of the residual is obtained. Then the initial guess value is incremented by a given value and the process repeated. The correct amount of the analyte component subtracted should be indicated by a minimum in the residuals ideally. Another way to determine when the correct subtraction has been achieved is by looking for negative residual values.

CHAPTER 4

RESULTS AND DISCUSSION

In this chapter, the results of various experimental procedures developed to achieve the proposed objectives and the methods used to quantitatively determine the concentration of the components in an unknown mixture simultaneously are tabulated and discussed. For the purpose of this research, simulated spectra and real experimental data were used. The simulated spectra were generated by using normal distribution. After all spectral parameters were generated simulated spectra were generated as discussed in Chapter 3. Two types of simulated spectra were generated; the first was a set of spectra that are broad and featureless and the second set have more structural and spectral features. Two-component mixtures were created from these base spectra with varying degrees of overlap. The simulated spectra generated were noise-free. A few examples of the generated spectra have been shown in Chapter 3.

Simulated Spectra

The simulated mixture data were made by multiplying the base spectra of component (I) and component (II) by a scalar value that represents the relative concentrations of the components with respect to the base spectra. Two sets of mixtures were created. The mixtures were made by keeping the concentration of one of the two components constant at base value, i.e. a relative concentration of 1.00, while the relative concentration of the other component is varied by multiplying the base spectra by a scalar. In addition to varying the relative concentrations of the simulated spectra, the degree of overlap between component (I) and component (II) was also varied gradually. Figures 5 and 6 show the spectra of the components and their mixtures. In Figure 5, the base spectrum of component (II) was shown along with the spectra of the different relative concentrations of component (I). In Figure 6, the resulting mixture spectra of component (II) with these different concentrations of component (I) are shown to indicate a minimal overlap between them.

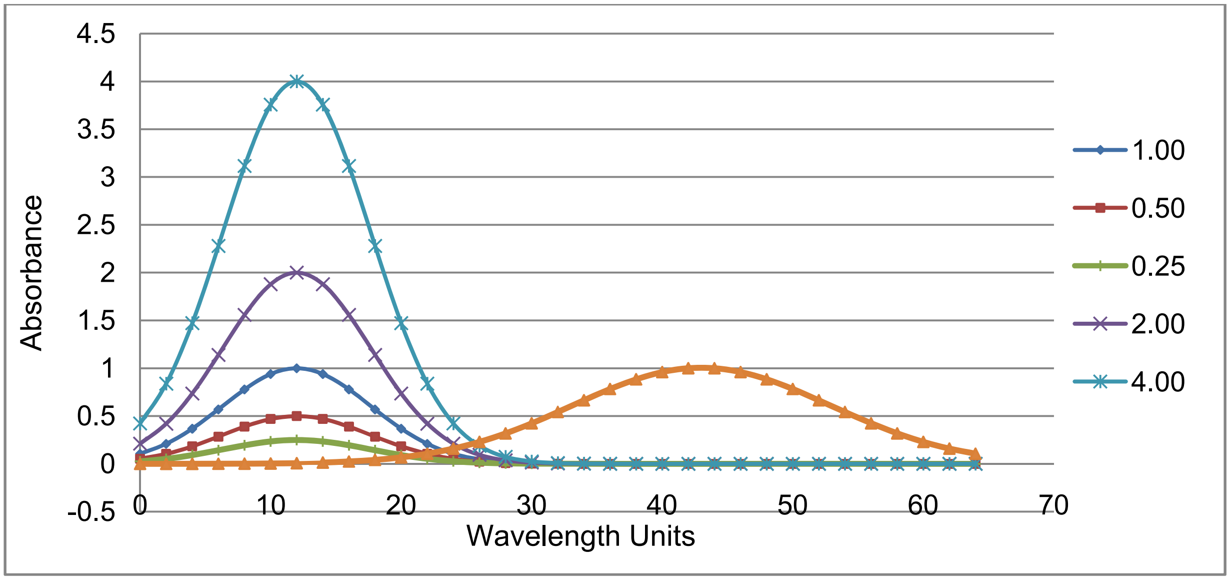

Figure 5. Simulated spectra of component (I) and component (II): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 multiples of the base spectrum.

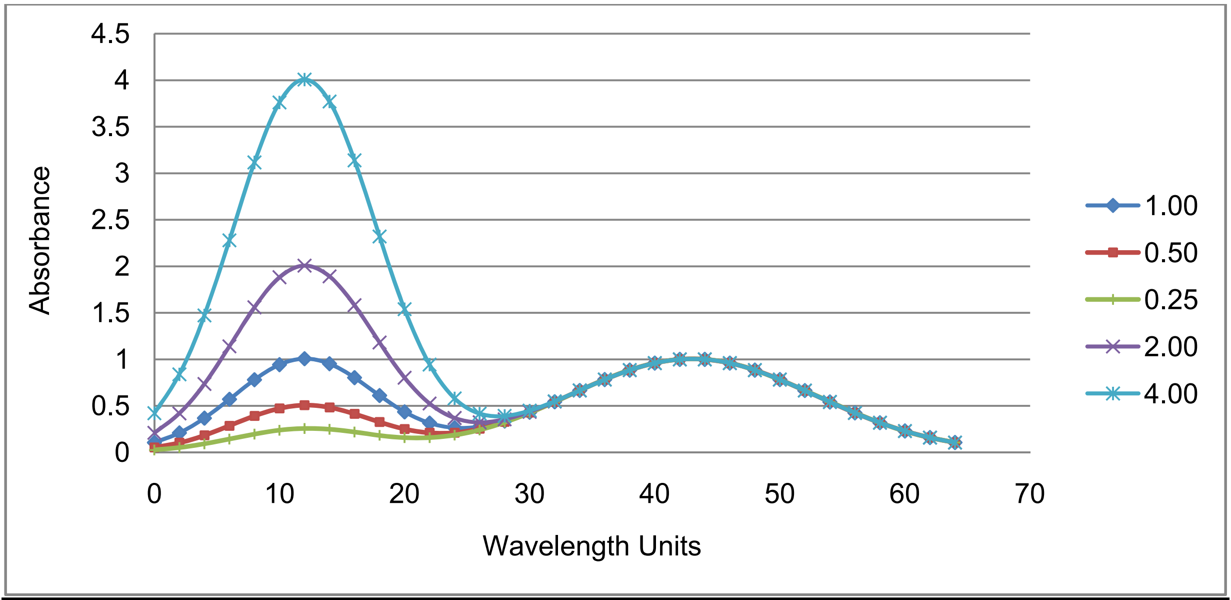

Figure 6. The spectra of mixtures of component (I) and component (II): keeping component (II) at a constant relative concentration of 1.00. The relative concentration of component (I) was varied from 0.25, 0.50, 1.00, 2.00, and 4.00 multiples of its base spectrum. Component (I) and component (II) do not overlap to any extent.

Simultaneous Equation Method on Components with Little Overlap

Component (I) and component (II) both have broad spectral features within regions 8-20 and 36-54 wavelength units, respectively. The two components have little or virtually no overlap. Their spectral peaks were well separated. The peak distribution of component (I) was mainly in the 8 to 20 wavelength units with peak maximum at the 12th wavelength unit. For component (II) the peak molar absorptivity occurs at 44th wavelength unit.

For calculation using simultaneous equation, we need to find wavelength units where one component absorbs strongly while another absorbs weakly. Because the two components are well separated spectrally, one cannot use the maximum of one in the analysis as the molar absorptivity of the other component would be zero. This will give erroneous results when solving the simultaneous equation.

Following the recommended procedure, calibration curves were made at selected wavelength units for the standard of component (I) and component (II). Molar absorptivities of component (I) and component (II) were obtained from the calibration curves made at selected wavelength units.

The results of solving simultaneous linear equation are shown in Table 2. The results show that when the analysis was done using wavelength units of 12 and 20, the relative concentration of component (I) found was 1.00 and that of component (II) was 1.02. At wavelength units of 26 and 30 the analysis gave the relative concentrations of 1.02 and 1.00, for component (I) and component (II), respectively. When the analysis was done at wavelength units 12 and 30, the calculated relative concentrations of component (I) and component (II) were 1.00 and 1.00, respectively. It can be seen that the results obtained for the two components when they were in a mixture of 1: 1 relative concentration were accurate as the overlap between the two components were minimal.

When the relative concentrations of component (I) was reduced to 0.25, at the wavelength units 12 and 20 the relative concentration of component (I) was accurate and that of component (II) was 1.01. The simulated spectra of these two components slightly

overlapped

Table 2. Method of simultaneous equation for the two-component mixtures with little overlap: Result of the simultaneous equation when the relative concentration of component (II) was kept at 1.00.

Expected Concentration of Component (I)	Component (I) Concentration Found	Component (II) Concentration found	Wavelength Units for Analysis
1.00	1.00	1.02	12, 20
	1.02	1.00	26,30
	1.00	1.00	12, 30
0.50	0.50	1.03	12, 20
	0.50	1.00	26,30
	0.50	1.00	12, 30
0.25	0.25	1.01	12, 20
	0.25	1.00	26,30
	0.25	1.00	12, 30
2.00	2.00	1.04	12, 20
	2.05	1.00	26,30
	2.00	1.00	12, 30
4.00	4.00	1.07	12, 20
	4.07	1.00	26,30
	4.00	1.00	12, 30

However, there were still regions in which each of the components was well separated. Thus one can still use this knowledge and find wavelength region where interference is minimal for use in analysis. The results of the simultaneous equation calculations show that at the selected wavelength units of 12 and 20, the relative concentration of component (I) found was 0.50 and that of component (II) was 1.03, at the wavelength units of 26 and 30, the relative concentrations found were 0.50 for component (I) and that of component (II) was 1.00. Using wavelength units of 12 and 30, the relative concentrations of component (I) found was 0.50 and that of component (II) was 1.00.

From the results obtained, it can be concluded that when there is little overlap even if one component is present at a lower concentration, the results obtained were still accurate. Similar results and conclusions can be made for the case where the relative concentration of component (I) was reduced to 0.25. When the relative concentration of component (I) was increased and was higher than that of component (II), the results obtained for both components were just as good. Some choices of wavelength units selected may give somewhat less accurate results, but, overall, the results obtained for the case when component (I) and component (II) were not overlapping to any extent were accurate, regardless of how one varies the relative concentrations.

Next, the relative concentration of component (II) was varied while that of component (I) was kept constant at a relative concentration of 1.00. Similar analysis was performed using the simultaneous equation method. The results of the analysis are tabulated in Table 3. As can be seen from the tabulated results, similar conclusions can be drawn as in the case where the relative concentration of component (I) was varied while keeping that of component (II) constant. Thus in general when the components have little spectral overlap, even if their spectra lack features and relative concentrations vary by quite a bit, the method of solving simultaneous equation with judicious choice of wavelength for simultaneous quantitative analysis can yield accurate results.

Table 3. Method of simultaneous equation for the two-component mixture with a little overlap: Result of the simultaneous equation when the relative concentration of component (I) was kept at 1.00.

Expected Concentration of Component (II)	Concentration of Component (II) Found	Concentration of Component (I) Found	Wavelength Units for Analysis
1.00	1.02	1.00	12, 20
	1.00	1.02	26,30
	1.00	1.00	12, 30
0.50	0.52	1.00	12, 20
	0.50	1.02	26,30
	0.50	1.00	12, 30
0.25	0.27	1.00	12, 20
	0.25	1.01	26,30
	0.25	1.00	12, 30
2.00	2.04	1.00	12, 20
	2.00	1.03	26,30
	2.00	1.00	12, 30
4.00	4.06	1.00	12, 20
	3.99	1.05	26,30
	4.00	1.00	12, 30

Method of Least-Squares on Components with Little Overlap

After using the method of solving simultaneous equation, the method of least-squares was tried. With this method all the data from all the wavelength units were used. The results of least-square analysis are tabulated in Table 4 and Table 5. From the tabulated data, one can see that the results obtained using the complete spectra by the method of least-squares

Table 4. Method of least-squares for the two-component mixtures with little overlap: Result of the method of least-squares when the relative concentration of component (II) was kept at 1.00 and all wavelength units were used.

Expected Concentration of Component (I)	Concentration of Component (I) Found	Concentration of Component (II) Found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

Table 5. Method of least-squares for the two-component mixtures with little overlap: Result of the method of least-squares when the relative concentration of component (I) was kept at 1.00 and all wavelength units were used.

Expected Concentration of Component (II)	Concentration of Component (II) Found	Concentration of Component (I) found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

gave very accurate results just as the method of solving simultaneous equation. Again, we can conclude that as long as there is little overlap, we can obtain good results under most circumstances and using different methods.

Simultaneous Equation Method when Severe Spectral Overlap is Present

Next, the effect of rather severe spectral overlap between components was studied. The same two components with featureless broad spectra were used but now overlap between them was introduced. The spectra of the components are shown in Figure 7 and their mixtures shown in Figure 8. In Figure 7, for component (I) the spectra of the different relative concentrations are overlaid while the spectrum of component (II) was kept at a relative concentration of 1.00. In Figure 8, one can see the resulting spectra of the mixtures when component (II) was added to the different concentrations of component (I). The features of component (I) at low concentrations (0.25 to 1.00) merge with that of component (II) and show little distinction between them. The results of solving simultaneous equations are tabulated in Tables 6 and Table 7. In Table 6, the relative concentration of component (I) was varied while that of component (II) was kept at 1.00. In Table 7, the reverse is true. Interestingly, the results obtained are accurate and seem to be just as good as when there was little overlap between the two components. From the results obtained one notes that even when the relative concentration of component (I) was not too much lower than that of component (II) the results were good. Even when component (I)'s relative concentration was reduced to 0.25, the results obtained was still good if the right wavelength unit was chosen for analysis. Similarly, one can also come to the same conclusion when the relative concentration of component (II) was lower than that of component (I). Thus, in general when the overlap is quite severe, the method of solving simultaneous equation with judicious choice of wavelength for analysis can yield good results as long as some rather distinct separation regions can be found. This is true in the case when there was no noise in the data. When noise is present the results might not be as good.

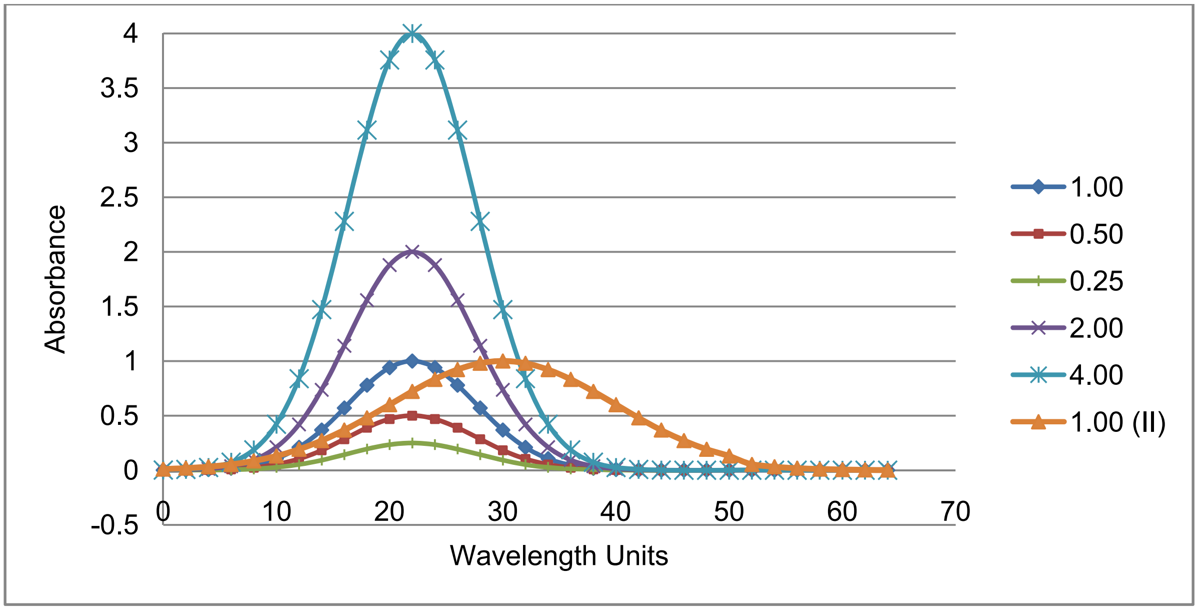

Figure 7. Simulated spectra of component (I) and component (II): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 multiples of the base spectrum.

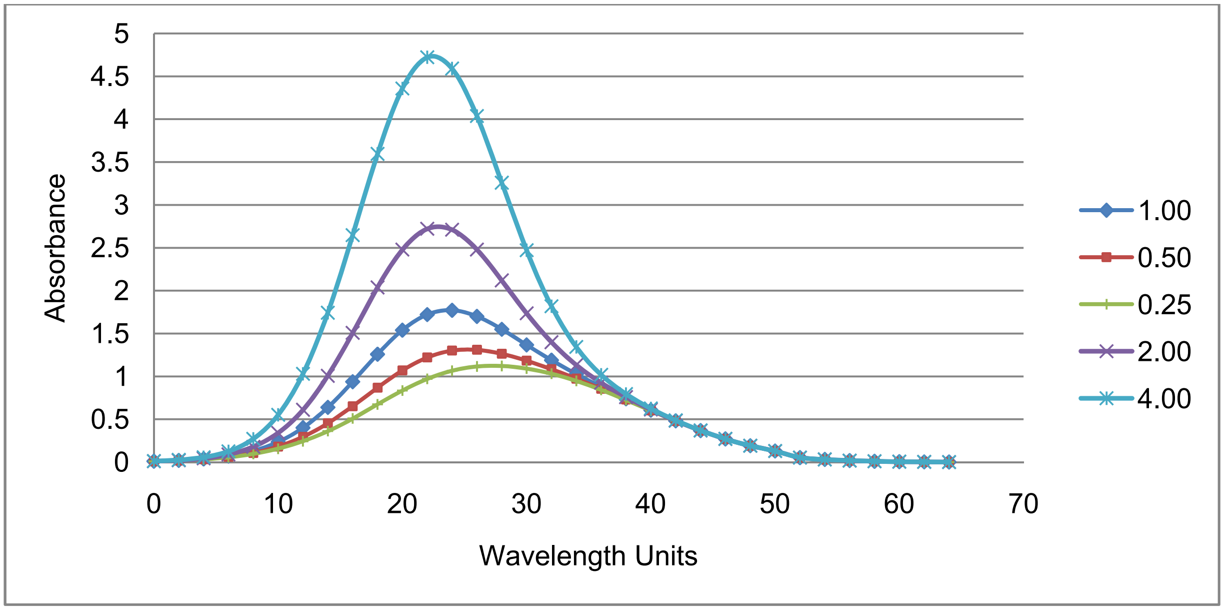

Figure 8. The spectra of mixtures of component (I) and component (II) (broad spectra): Keeping component (II) at a constant relative concentration of 1.00. The relative concentration of component (I) was varied from 0.25, 0.50, 1.00, 2.00, and 4.00 multiples of its base spectrum. Component (I) and component (II) overlap severely.

Table 6. Method of simultaneous equation for the two-component mixtures with more severe overlap: Result of the simultaneous equation when the relative concentration of component (II) was kept at 1.00

Expected Concentration of Component (I)	Concentration of Component (I) Found	Concentration of Component (II) found	Wavelength Units for Analysis
1.00	0.99	1.00	12, 36
	1.00	1.00	22, 30
	0.99	1.01	12, 30
0.50	0.52	0.97	12, 36
	0.50	1.00	22, 30
	0.49	1.01	12. 30
0.25	0.24	1.00	12, 36
	0.25	1.00	22, 30
	0.24	1.01	12, 30
2.00	1.99	1.00	12, 36
	2.00	1.00	22, 30
	1.98	1.01	12, 30
4.00	3.98	1.00	12, 36
	3.99	1.01	22, 30
	3.97	1.01	12, 30

Table 7. Method of simultaneous equation for the two-component mixtures with more severe overlap: Result of the simultaneous equation when the relative concentration of component (I) was kept at 1.00

Expected Concentration of Component (II)	Concentration of Component(II) Found	Concentration of Component (I) found	Wavelength Units for Analysis
1.00	1.00	0.99	12, 36
	1.00	1.00	22, 30
	1.01	0.99	12, 30
0.50	0.50	0.99	12, 36
	0.50	1.00	22, 30
	0.50	0.99	12. 30
0.25	0.25	1.00	12, 36
	0.25	1.00	22, 30
	0.25	0.99	12, 30
2.00	2.00	0.98	12, 36
	2.00	1.00	22, 30
	2.01	0.97	12, 30
4.00	4.00	0.97	12, 36
	4.00	0.99	22, 30
	4.02	0.95	12, 30

Method of Least-Squares when Severe Spectral Overlap is Present

The same set of data was subjected to the analysis by the method of least-squares. The results obtained are tabulated in Table 8 and 9. The method of least-squares employed the data from the whole spectral range. The method of least-squares yields accurate results in all cases, even better than those obtained from solving simultaneous equations. Thus a rather severe degree of overlap does not seem to affect the results at all. This is most likely due to the fact that these data were error free and thus with judicious choice of wavelengths chosen in the case of simultaneous equation method or with the method of least-squares, the results are equally good.

Table 8. Method of least-squares for the two-component mixtures with more overlap: Result of the least-squares method when the relative concentration of component (II) was kept at 1.00 and all wavelength units were used.

Expected Concentration of Component (I)	Concentration of Component (I) Found	Concentration of Component (II) Found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

Table 9. Method of least-squares for the two-component mixtures with more overlap: Result of the method of least-squares when the relative concentration of component (I) was kept at 1.00 and all wavelength units were used.

Expected Concentration of Component (II)	Concentration of Component (II) Found	Concentration of Component (I) Found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

Component with Greater Spectral Features

After studying the components with broad featureless spectra, how their degree of overlap, and how their relative concentration ratios in the mixture may affect their simultaneous quantitative analysis, attention is now focused on components with more structured spectral features. Instead of having just simple single broad peak spectrum, now more peaks are present in the spectra of the components under study. These spectra are shown in Figure 9 to Figure 12. We observed from the calculations that by using the method of solving simultaneous equations and the method of least-squares the results were accurate in most instances, with greater structural features.

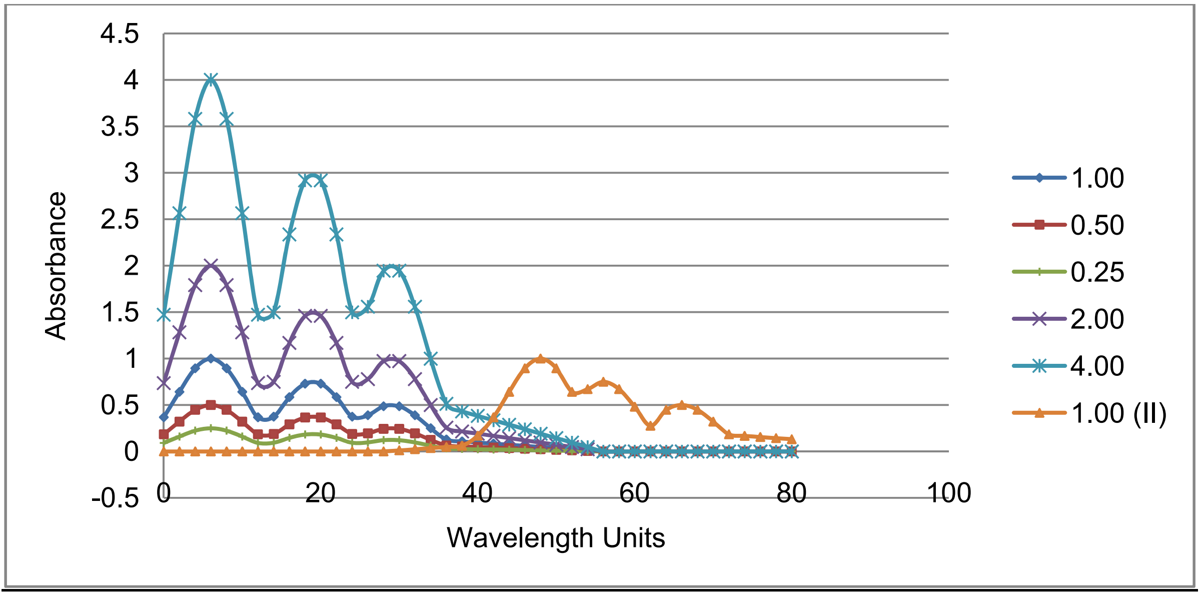

Figure 9. Simulated featured spectra of component (I) and component (II) (less overlap): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 multiples of the base spectrum. Component (I) and component (II) do not overlap to any extent.

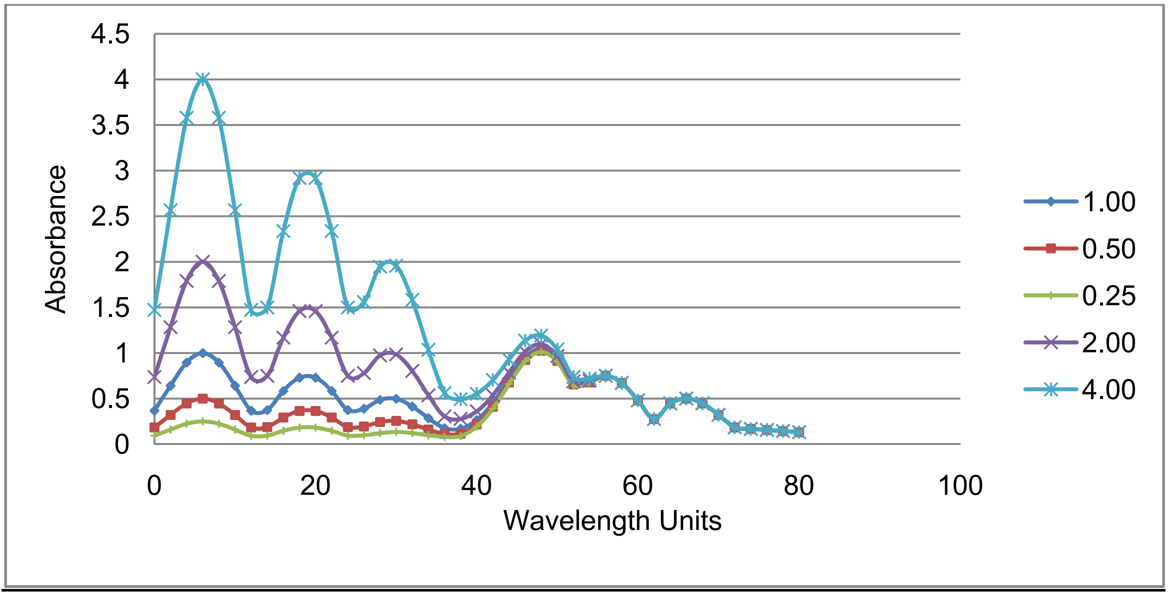

Figure 10. Mixture spectra of the two featured simulated components: Component (I) and component (II), with component (II) kept at a constant relative concentration of 1.00. The relative concentration of component (I) was varied from 0.25, 0.50, 1.00, 2.00, and 4.00 of its base spectrum. Component (I) and component (II) do not overlap to any extent.

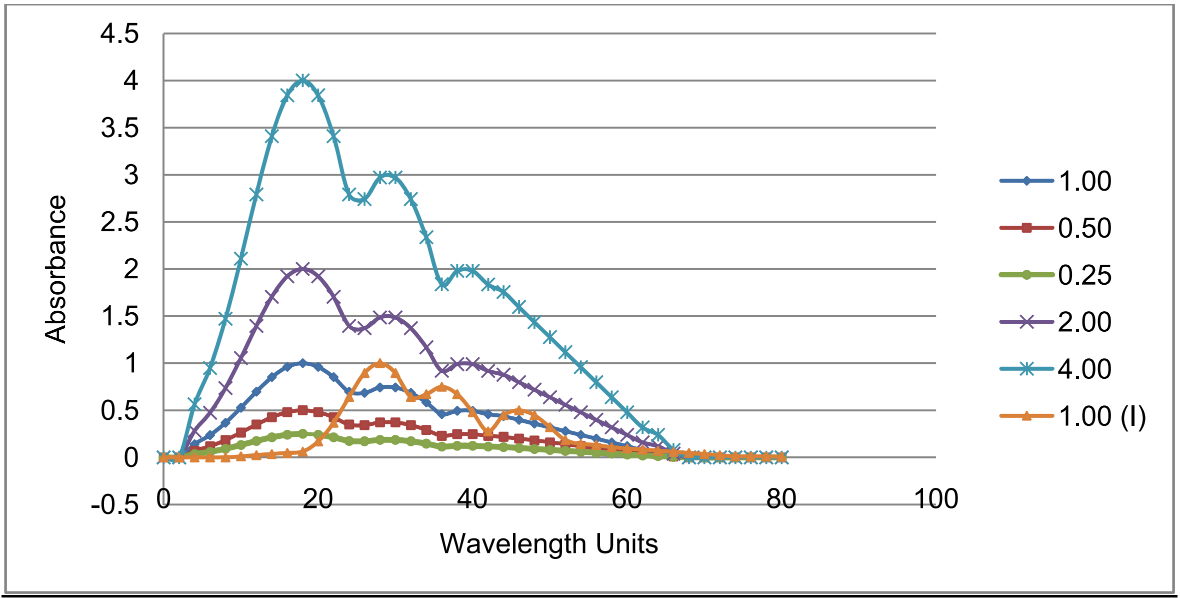

Figure 11 Simulated featured spectra of component (I) and component (II) (severe overlap): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 multiples of the base spectrum. Component (I) and component (II) overlap severely.

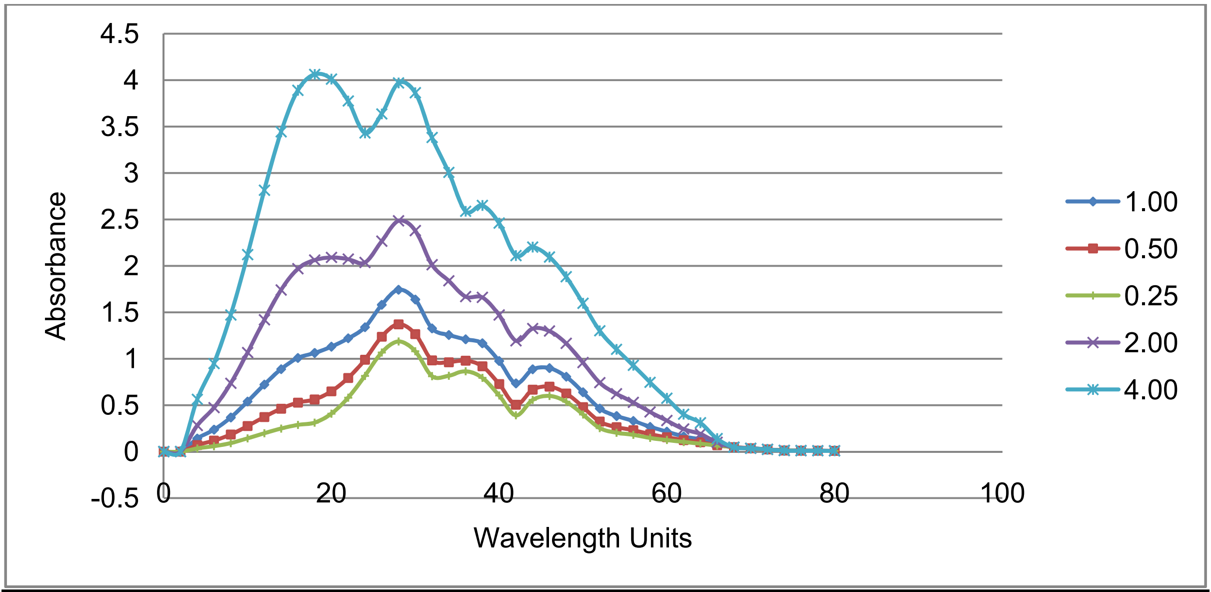

Figure 12 Mixture spectra of the two featured simulated components: Component (I) and component (II), with component (II) kept at a constant relative concentration of 1.00. The relative concentration of component (I) was varied from 0.25, 0.50, 1.00, 2.00, and 4.00 multiples of its base spectrum. Component (I) and component (II) overlap severely.

Method of Solving Simultaneous Equation

The results of simultaneous quantitative analyses of the mixtures of two more structured components are shown in Table 10 and Table 11. The concentration of one of the components was kept constant at a relative concentration of 1.00 while that of the other was varied. Table 10 shows an interesting case of a consistent, poor result for a component with a certain choice of wavelength units. That is the calculated relative concentrations are either too high or too low. In instances when wavelength units of 28 and 48 are used the result obtained for component (I) was consistently less accurate (lower) than the other wavelength choices. This choice also showed consistently higher results for component (II). As can be seen in Table 10, the calculated results for component (I) were overall good. It is also observed that when the concentration of component (II) is lower than that of component (I), its concentrations were not as good, especially when the wavelength units chosen were 28, 48. When the wavelength units 18, 28 were employed, the calculated concentrations of component (II) were accurate. Thus it seems that the correct choice of wavelength units used for the method of solving simultaneous equation is critical for the component under consideration. In Table 10, the wavelength units, chosen were optimized for component (I) mostly, with only wavelength unit 48 being the one where component (II) absorbs strongly by itself. Using the same set of wavelength units, Table 11 shows the same results were obtained when the relative concentration of component (II) was varied. Again, the results obtained using wavelength units of 25, 48 were not as good as the other wavelength units chosen. This observation was consistent for the relative calculated concentrations of both component (I) and component (II). Wavelength unit 28 was the weakest peak of component (I) while wavelength units 12 and 18 were the major peaks of component (I). This error is much bigger when the relative concentration of component (I) is much smaller than that of component (II). Thus, from the set of results, choice of wavelength units and the relative concentration of the components in the mixture do have an effect on the calculated results.

Table 10. Method of simultaneous equation for the two-component mixtures with more structured spectral features and greater degree of overlap: The relative concentration of component (II) was kept at 1.00.

Expected Concentration of Component (I)	Concentration of Component (I) Found	Concentration of Component (II) found	Wavelength Units for Analysis
1	1.00	0.99	12, 18
	0.94	1.05	28, 48
	1.00	1.00	18, 28
0.5	0.50	1.01	12, 18
	0.46	1.03	28, 48
	0.50	1.00	18, 28
0.25	0.25	1.02	12, 18
	0.22	1.02	28, 48
	0.25	1.01	18, 28
2	2.00	0.96	12, 18
	1.90	1.08	28, 48
	2.00	1.00	18, 28
4	4.01	0.89	12, 18
	3.82	1.14	28, 48
	4.00	1.00	18, 28

Table 11. Method of simultaneous equation for the two-component mixtures with more structured spectral features and greater degree of overlap: The relative concentration of component (I) was kept at 1.00.

Expected Concentration of Component (II)	Concentration of Component (II) Found	Concentration of Component (I) Found	Wavelength Units for Analysis
1.00	0.99	1.00	12, 18
	1.05	0.94	28, 48
	1.00	1.00	18, 28
0.50	0.48	1.00	12, 18
	0.54	0.95	28, 48
	0.50	1.00	18, 28
0.25	0.22	1.00	12, 18
	0.28	0.96	28, 48
	0.25	1.00	18, 28
2.00	2.02	1.00	12, 18
	2.06	0.92	28, 48
	2.00	1.00	18, 28
4.00	4.07	0.99	12, 18
	4.09	0.88	28, 48
	4.00	1.00	18, 28

Method of Least-Squares

The results of the method of least-squares on the two component mixtures with more structured spectral features are given in Table 12 and Table 13. In Table 12, the relative concentration of component (I) were varied while that of component (II) was kept at a constant value of 1.00. In Table 13, the relative concentration of component (II) is now varied while that of component (I) is kept constant at 1.00. As can be seen, the results in both cases were accurate with no error at all. Even when the degree of overlap was increased, as shown in Table 14 and Table 15, the results obtained using the method of least-squares were equally good. Thus it seems that, when there was no noise in the data and that there was good spectral features in the components under study, the method of least-squares yield accurate simultaneous quantitative results regardless of degree of overlap or relative concentration in the range under study.

Table 12. Method of least-squares for the two-component mixtures with more structured spectral feature and small degree of overlap: The relative concentration of component (II) was kept constant at 1.00 and all wavelength units were used.

Expected Concentration of Component (I)	Concentration of Component (I) Found	Concentration of Component (II) found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

Table 13. Method of least-squares for the two-component mixtures with more structured spectral features and a small degree of overlap: The relative concentration of component (I) was kept constant at 1.00 and all wavelength units were used.

Expected Concentration of Component (II)	Concentration of Component (II) Found	Concentration of Component (I) found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

Table 14. Method of least-squares for the two-component mixtures with more structured spectral features and a greater degree of overlap: The relative concentration of component (II) was kept constant at 1.00 and all wavelength units were used.

Expected Concentration of Component (I)	Concentration of Component (I) Found	Concentration of Component (II) found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

Table 15. Method of least-squares for the two-component mixtures with more structured spectral features and a greater degree of overlap: The relative concentration of component (I) was kept constant at 1.00 and all wavelength units were used.

Expected Concentration of Component (II)	Concentration of Component (II) Found	Concentration of Component (I) found
1.00	1.00	1.00
0.50	0.50	1.00
0.25	0.25	1.00
2.00	2.00	1.00
4.00	4.00	1.00

Simultaneous Quantitative Analysis of Experimental Data

The concentrations of Fe(III) and Cu(II) azide complexes were quantitatively determined in a mixture with varying volume ratios of 1:1, 2:1, and 3:1 of iron(III) and copper(II) azide complex working solutions and vice versa. The molar ratio of iron(III) and the sodium azide was a 1: 1 ratio and that of the copper(II) and the sodium azide was also a 1:1 ratio. Acetone was used because it is one of the most widely used solvents and is miscible with water. It also enhanced the intensities of iron(III) and copper(II) colors. The solutions were prepared under the same experimental condition to ensure consistency in spectral measurements. Following the recommended procedure, calibration curves were made at selected wavelengths for the standard solutions of iron(III) and copper(II) azide complexes. Molar absorptivities of iron(III) and copper(II) azides were obtained from the calibration curves made at selected wavelengths.

The concentration of iron(III) and copper(II) azide complex solutions were made below 10^{-4} M to ensure accuracy and precision of the data and also to make sure Beer Lambert's law was obeyed. Figure13 shows the spectra of 8.0 x 10^{-5} M iron(III) and 4.0 x 10^{-5} M copper(II) azide complexes. From the spectra in Figure 13 it was observed that there are some regions where iron(III) absorbs strongly while that of copper(II) is small and vice versa.

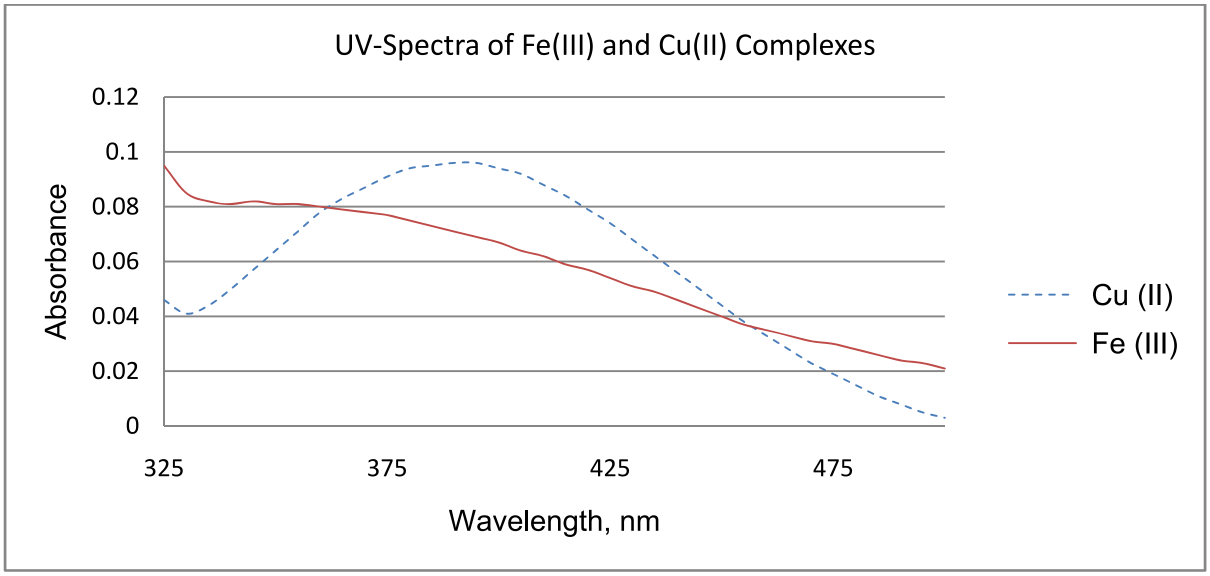

Figure 13. UV- absorption spectra of Fe(III) and Cu(II) azide complexes: With concentration of 0.08 mM and 0.04 mM, respectively.

Based on these observations, six wavelengths were selected. The wavelengths selected for the analyses were 330, 345, 365, 400, 445, and 485 nm. The selection of the wavelengths was focused on regions where the contribution of iron(III) was greater and that of copper(II) was small and similarly in regions where copper(II) absorbs strongly while that of iron(III) was weak.

Three set of mixtures with known concentration of the individual working standard solutions used were prepared in the working solutions volume ratio; 1:1, 2:1, and 3:1 of iron(III) and copper(II) azide complexes and vice versa. The spectra of the mixtures in which iron(III) is present at higher relative concentrations are shown in Figure 14 and those with copper(II) at higher relative concentrations are shown in Figure 15. The concentrations of iron(III) and copper(II) azide complexes present in the mixture were then quantitatively determined by the method of solving simultaneous linear equation and the method of least squares. The method of simultaneous equation determination of the concentrations were done to compare the known simplest and the more sophisticated chemometric methods, in quantitative analysis of multi-component data, and to know and understand the capabilities and shortcomings in analysis of

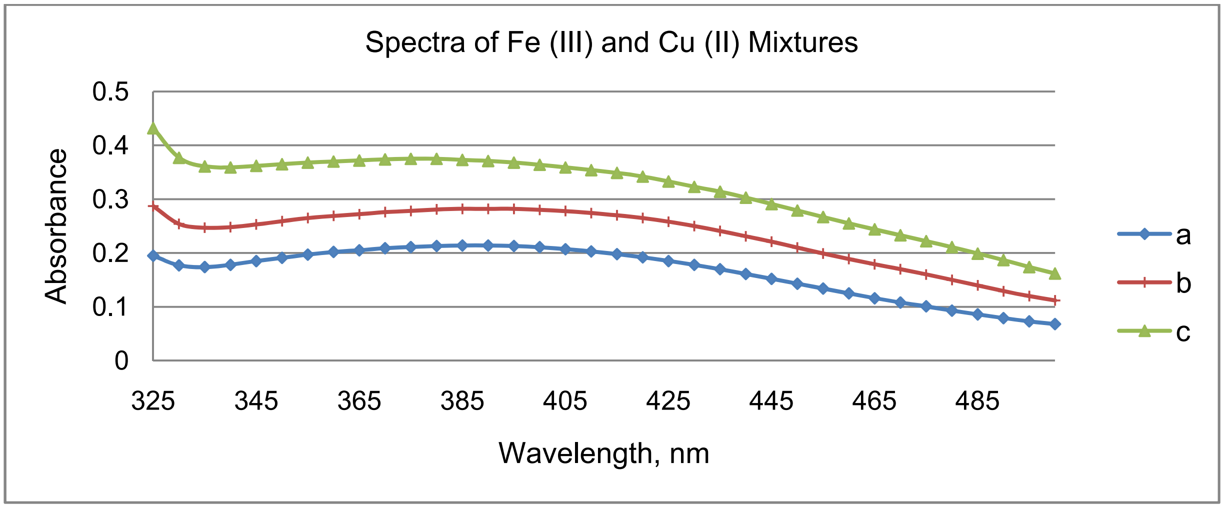

Figure 14. Spectra of Fe(III) and Cu(II) azide complexes: Concentration ratio of the spectra are; for (a) 0.08 mM: 0.04 mM, (b) 0.16 mM: 0.04 mM and (c) 0.24 mM: 0.04 mM respectively, of iron(III) azide complex: copper(II) azide complex.

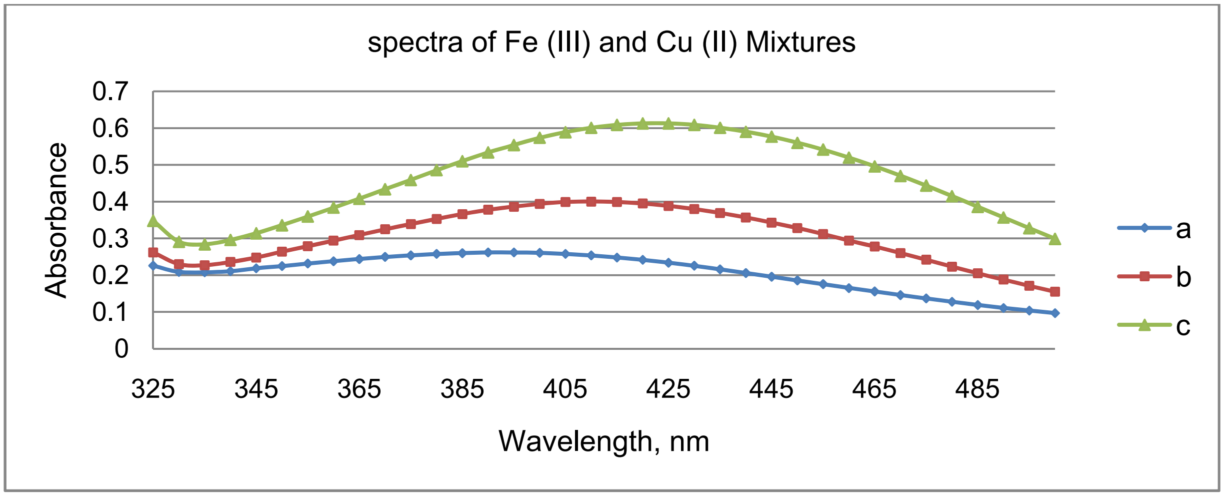

Figure 15. Spectra of Fe(III) and Cu(II) azide complexes: Concentration ratio of the spectra are; for (a) 0.08 mM: 0.04 mM, (b) 0.08 mM: 0.08 mM and (c) 0.08 mM: 0.12 mM respectively, of iron(III) azide complex and copper(II) azide complex.

mixture data with different degree of overlap. Results of the simultaneous linear equation and the method of least squares, used in the simultaneous determination of the concentrations of iron(III) and copper(II) azides present in the mixtures with varying concentration ratios are given in Table 16 and Table 17.

The mixture of 8.0 x 10^{-5} M iron(III) and 4.0 x 10^{-5} M copper(II) azide complex shows a broad absorption band in the region 350 nm to 415 nm. From the individual spectrum of 8.0 x 10^{-5} M iron(III) and 4.0 x 10^{-5} M copper(II) azide complex solution, it can easily be observed that the two absorption spectra of the two complexes had substantial overlap. However, there were some small regions in which the components were free of overlap absorption. The iron(III) strongly absorbs in the following regions; 330 nm, 345 nm, 365 nm, and 485 nm while that of copper(II) was low in these regions. Copper(II) azide complex absorbs strongly at 400 nm and 445 nm, while that of iron(III) azide complex was low.

Method of Simultaneous Equation

The results from the simultaneous linear equation, given in Tables 16 and Table 17, showed that the calculated concentrations of iron(III) and copper(II) azide complexes present in the mixture were much lower compared to the expected values. In Table 16, the percent errors were off by about 0.4% to 96.3%. The most significant error occurred when 365, 445 nm and 345, 485 nm were chosen for analysis. At these wavelengths for the 1:1 volume ratio mixture the degree of overlap was large with percent error of 96.3 % for iron(III) and 77.8% for copper(II), while percent error of iron(III) and copper(II) calculated were 77.5 % and 86.8% respectively at 365, 445 nm, and 345, 485 nm. At 330 and 400 nm the percent errors of calculated concentration were also lower. The results for iron(III) got progressively better as its volume ratio was increased with respect to copper(II). In fact, results obtained by using 330 and 400 nm for analysis were consistently better for both components than the other two choices. Using this wavelength when the working solution volume ratio of the iron(III) and copper(II) were 3: 1, one obtained the best and accurate results.

Table 16. Method of simultaneous equation for two-component mixtures of Fe(III) and Cu(II) azide working solutions: The volume ratio of the working solutions are 1:1, 2:1, and 3:1.

WORKING SOLUTIONS VOLUME RATIO	PRESENT		FOUND		WAVELENGTH λ (nm)
	Fe(III)	Cu (II)	Fe(III)	Cu (II)	
1:1	8.0 x 10^{-5}	4.0 x 10^{-5}	1.02 x 10^{-4} (27.5 %)	2.85 x 10^{-5} (28.8 %)	330, 400
			1.57 x 10^{-4} (96.3 %)	8.90 x 10^{-6} (77.8 %)	365, 445
			1.42 x 10^{-4} (77.5 %)	5.28 x 10^{-6} (86.8 %)	345, 485
2:1	1.6 x 10^{-4}	4.0 x 10^{-5}	1.53 x 10^{-4} (4.4 %)	3.32 x 10^{-5} (17.0 %)	330, 400
			1.99 x 10^{-4} (24.4 %)	1.74 x 10^{-5} (56.5 %)	365, 445
			1.86 x 10^{-4} (16.3 %)	1.47 x 10^{-5} (63.3 %)	345, 485
3:1	2.4 x 10^{-4}	4.0 x 10^{-5}	2.41 x 10^{-4} (0.4 %)	3.19 x 10^{-5} (20.2 %)	330, 400
			2.78 x 10^{-4} (15.8 %)	2.05 x 10^{-5} (48.8 %)	365, 445
			2.67 x 10^{-4} (11.3 %)	2.06 x 10^{-5} (48.5 %)	345, 485

Table 17. Method of simultaneous equation for the two-component mixtures of Fe(III) and Cu(II) azide working solutions: The volume ratio of the working solutions are; 1:1, 1:2, and 1:3.

WORKING SOLUTIONS VOLUME RATIO	PRESENT		FOUND		WAVELENGTH λ (nm)
	Fe(III)	Cu (II)	Fe(III)	Cu (II)	
1:1	8.0 x 10^{-5}	4.0 x 10^{-5}	1.02 x 10^{-4} (27.5 %)	2.85 x 10^{-5} (28.8 %)	330, 400
			1.57 x 10^{-4} (96.3 %)	8.90 x 10^{-6} (77.8 %)	365, 445
			1.42 x 10^{-4} (77.5 %)	5.28 x 10^{-6} (86.8 %)	345, 485
1:2	8.0 x 10^{-5}	8.0 x 10^{-5}	1.02 x 10^{-4} (27.5 %)	7.74 x 10^{-5} (2.8 %)	330, 400
			1.79 x 10^{-4} (123.8 %)	4.63 x 10^{-5} (42.1 %)	365, 445
			1.59 x 10^{-4} (98.8 %)	3.70 x 10^{-5} (53.8 %)	345, 485
1:3	8.0 x 10^{-5}	1.2 x 10^{-4}	1.09 x 10^{-4} (36.3 %)	1.23 x 10^{-4} (2.5 %)	330, 400
			1.74 x 10^{-4} (117.5 %)	9.69 x 10^{-5} (19.3 %)	365, 445
			1.57 x 10^{-4} (96.3 %)	8.90 x 10^{-5} (11.3 %)	345, 485

The most significant error consistently occurred at wavelength 365, 445 and 345, 485 nm.

Next the volume ratio of iron(III) and copper(II) azide complex working solutions were varied, keeping the volume of iron(III) constant at 1 mL. Similar analyses were performed using the simultaneous equation method. The results of the analysis are shown in Table 17. Again, it seems that although the results in general are not satisfactory, the results obtained using wavelength pair of 330, and 400 nm seem to be the most acceptable. In general when the volume of copper(II) azide was higher than that of iron(III) azide the results were poor. This is quite understandable because of the signal strength of iron(III) and also its spectrum being so broad and absolutely featureless without a peak in the wavelength region where measurements were made.

Thus, when the component in the mixture has broad featureless spectra, severely overlapping and with errors present in the real data as opposed to no error from simulated data, the calculated results using simultaneous equation suffered greatly. In this very difficult situation, the choice of wavelengths for analysis can help critically. But there are far fewer choices. This can also be attributed to the fact that the wavelength regions of least interference are also wavelength regions where the components absorb weakly and thus measurements errors were maximal.

Method of Least-Squares Analysis

The method of least-squares using the complete spectra was now applied to the same set of data. The results from the method are shown in Table 18 and Table 19. The results obtained were an improvement as compared to that from the method of solving simultaneous equations. From the tabulated data, one can see that the results obtained using the wavelengths 325 to 415 nm by applying the method of least-squares gave better results. The results for both iron(III) and copper(II) simultaneously were best when their concentrations were about equal and thus their signal strength was about the same. From Table 18, it can be seen that the calculated concentrations for iron(III) was higher in the1:1 volume ratio with percent error of 45.0% and as the volume of iron(III) was increased the percent error decreased from 10.6% to 5.8%, while the calculated concentrations of copper(II) was higher in the 1:1 volume ratio with percent error of about 17%, and as the volume of copper(II) was increased the percent error in the 2:1 volume ratio was 30.8% and for the 3:1 volume ratio the percent error was 22.5%.

Table 18. Method of least-squares for the volume ratios of Fe(III) and Cu(II) azide complexes in the two-component mixture: Varying the volume of Fe(III) azide complex.

WORKING SOLUTIONS VOLUME RATIO	PRESENT		FOUND		WAVELENGTH λ (nm)
	Fe(III)	Cu(II)	Fe(III)	Cu(II)	
1 :1	8.0 x 10^{-5}	4.0 x 10^{-5}	1.16 x 10^{-4} (45.0 %)	4.68 x 10^{-5} (17.0 %)	325-415
2 :1	1.6 x 10^{-4}	4.0 x 10^{-5}	1.43 x 10^{-4} (10.6 %)	5.23 x 10^{-5} (30.8 %)	
3 :1	2.4 x 10^{-4}	4.0 x 10^{-5}	2.26 x 10^{-4} (5.8 %)	4.90 x 10^{-5} (22.5 %)	

Table 19. Method of least-squares for the volume ratios of Fe(III) and Cu(II) azide complexes in the two-component mixtures: Varying the volume of Cu(II).

WORKING SOLUTIONS VOLUME RATIO	PRESENT		FOUND		WAVELENGTH λ (nm)
	Fe(III)	Cu(II)	Fe(III)	Cu(II)	
1 :1	8.0 x 10^{-5}	4.0 x 10^{-5}	1.16 x 10^{-4} (45.0 %)	4.68 x 10^{-5} (17.0 %)	325-415
1 :2	8.0 x 10^{-5}	8.0 x 10^{-5}	3.31 x 10^{-5} (58.6 %)	1.23 x 10^{-4} (53.8 %)	
1 :3	8.0 x 10^{-5}	1.2 x 10^{-4}	1.07 x 10^{-5} (86.6 %)	1.72 x 10^{-4} (43.3 %)	

The results obtained when copper(II) was varied to higher volumes, thus with greater absorbance value, were much worse than for the case when iron(III) was higher. In fact the results in Table 19 were in general much worse than in Table 18.

Method of Total Spectral Subtraction

In light of the difficulty of the method of solving simultaneous equation and the method of least squares on experimental data of mixtures whose components overlap severely, another method was explored and attempted. The method of total subtraction is similar to that of the rank annihilation factor analysis (31) method. However, it is a much simpler approach without needing all the complicated, highly sophisticated and expensive software packages. The rational is that if one knows one or several of the components present in a multi-component mixture and thus have the standard spectra of these known components, one can subtract out by an iterative procedure the known components' spectral information from the mixture.

The major difficulty of the method is how one determines when the correct amount of the standard has been subtracted out. In this study, a single way that was tried was simply to calculate the sum of squares of residuals after each iterative subtraction. When the sum is a minimum, the subtraction is assumed to be complete.

The method was tried on the first set of data that was used for the other two methods. The mixture was those of components with virtually no overlap as shown in Figure 5. The concentration of one component was varied, while the other component was kept at 1.00 as was done before. The calculated minimum is shown in Table 20 and the plots of the minimum found are shown in Figure 16. The calculated concentrations based on the total spectral subtraction method are shown in Table 21. As can be seen, the results are accurate.

Similarly, the method was applied to the mixture with components that have structured spectra each with three peaks as shown in Figure 9. The calculated minimum is shown in Table 22, the plot of the minima is shown in Figure 17, and the results of the concentration obtained are shown in Table 23. Again, when there is little overlap, the results are accurate, comparing them to the expected concentrations.

Table 20. Calculated minima for component (I) in the two-component mixture with little overlap (broad spectrum): The relative concentration of component (II) was kept at 1.00

1.00		0.50		0.25		2.00		4.00	
Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.
0.80	9.12	0.20	9.40	0.01	9.23	1.70	9.40	3.50	10.26
0.85	8.94	0.25	9.03	0.20	8.88	1.95	8.88	3.80	9.12
0.95	8.86	0.26	9.00	0.25	8.86	2.00	8.86	3.90	8.94
1.00	**8.85**	**0.45**	**8.86**	**0.26**	**8.85**	**2.01**	**8.85**	4.00	8.86
1.01	**8.85**	**0.50**	**8.86**	0.45	9.00	**2.05**	**8.85**	**4.01**	**8.85**
1.03	8.88	0.60	8.88	0.50	9.09	2.10	8.88	**4.03**	**8.85**
1.20	9.22	0.80	9.36	0.61	9.36	2.40	8.89	4.50	9.96

Rel. =Relative, Conc. =Concentration, Sq. = Square

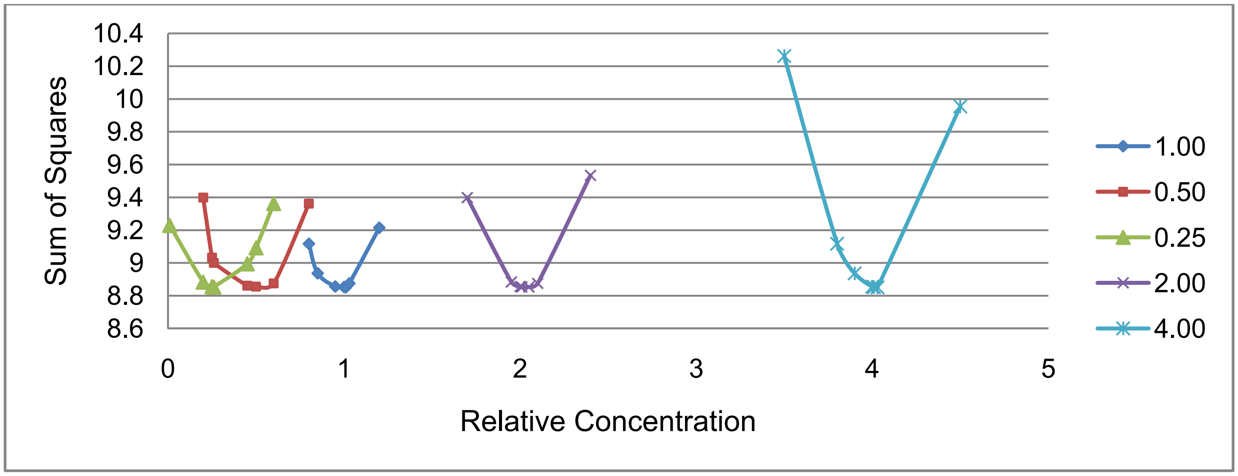

Figure. 16. Plots of calculated minima of sum of squares (broad spectrum): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 of the base spectrum with less overlap.

Table 21. Calculated concentrations for component (I) in the two-component mixture with a little degree of overlap (broad spectrum): The relative concentration of component (II) was kept at 1.00

Expected Concentration of Component (I)	Concentration of Component (I) Found
1.00	1.01
0.50	0.48
0.25	0.26
2.00	2.03
4.00	4.02

Table 22. Calculated minima for component (I) in the two-component mixture with a little degree of overlap (structured spectrum): The relative concentration of component (II) was kept at 1.00

1.00		0.50		0.25		2.00		4.00	
Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.
0.90	6.37	0.30	6.62	0.22	6.27	1.80	6.62	3.80	6.62
0.95	6.29	0.40	6.37	0.23	6.26	1.90	6.37	3.90	6.37
1.00	6.24	0.45	6.29	0.24	6.25	1.95	6.29	3.95	6.29
1.01	6.24	0.50	6.24	0.25	6.24	2.00	6.24	4.00	6.24
1.02	**6.23**	0.51	6.24	0.26	6.24	2.01	6.24	4.01	6.24
1.03	**6.23**	**0.52**	**6.23**	**0.27**	**6.23**	**2.02**	**6.23**	**4.02**	**6.23**
1.09	6.25	**0.55**	**6.23**	**0.29**	**6.23**	2.10	6.25	4.10	6.25
1.10	6.25	0.70	6.39	0.33	6.24	2.20	6.39	4.20	6.39
1.20	6.39	0.80	6.67	0.36	6.26	2.30	6.67	4.30	6.67

Rel. =Relative, Conc. =Concentration, Sq. = Square

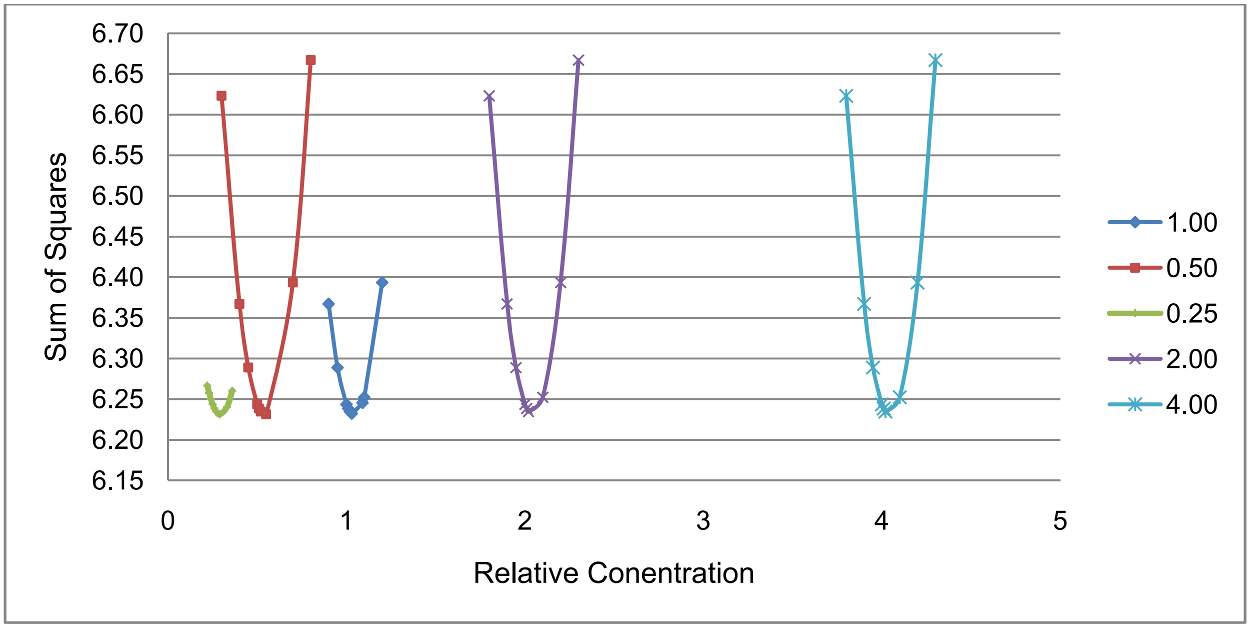

Figure 17. Plots of calculated minima of sum of squares (structured spectrum): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 of the base spectrum, with little overlap.

Table 23. Calculated concentrations for component (I) in the two-component mixture with a little degree of overlap (structured spectrum): The relative concentration of component (II) was kept at 1.00

Expected Concentration of Component (I)	Concentration of Component (I) Found
1.00	1.03
0.50	0.54
0.25	0.28
2.00	2.02
4.00	4.02

However, when the method was applied to the severely overlapped mixture of Figure 7 with components that have broad and featureless spectra, the results obtained were always high. The calculated minimum is shown in Table 24 and the plots of the calculated minima are shown in Figure 18. Because of the overlap, the resulting leftover mixture after subtraction still has rather large values of absorbances in the overlapping region. Thus, the residuals do not reach a minimum at the correct instance, higher than expected calculated concentrations were the results as shown in Table 25.

Similarly, the method was applied to the mixture with components that have structured spectra as shown in Figure 11. The calculated minimum is shown in Table 26 and the plot of the minima is shown in Figure 19 and the results of the concentration obtained are shown in Table 27. Again, the overlapping spectra present problems. One needs to find a better quantitative measure to signal the "end point", so to speak, of the subtraction, although one could visually see through the plots of the left over spectra that the subtraction was correctly done.

Table 24. Calculated minima for component (I) in the two-component mixture with a severe degree of overlap (broad spectrum): The relative concentration of component (II) was kept at 1.00

1.00		0.50		0.25		2.00		4.00	
Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.
0.90	9.78	0.45	9.26	0.24	8.86	1.95	9.26	3.80	10.89
1.00	8.77	0.50	8.77	0.25	8.77	2.00	8.77	4.00	8.77
1.01	8.67	0.51	8.67	0.26	8.67	2.02	8.58	4.02	8.58
1.60	4.80	**1.30**	**4.27**	0.60	6.01	2.50	5.21	4.20	7.04
1.90	**4.16**	2.30	7.68	**1.20**	**4.15**	**3.00**	**4.15**	**5.00**	**4.15**
3.00	9.57	2.50	9.57	2.10	8.12	4.00	9.52	6.00	9.57

Rel. =Relative, Conc. =Concentration, Sq. = Square

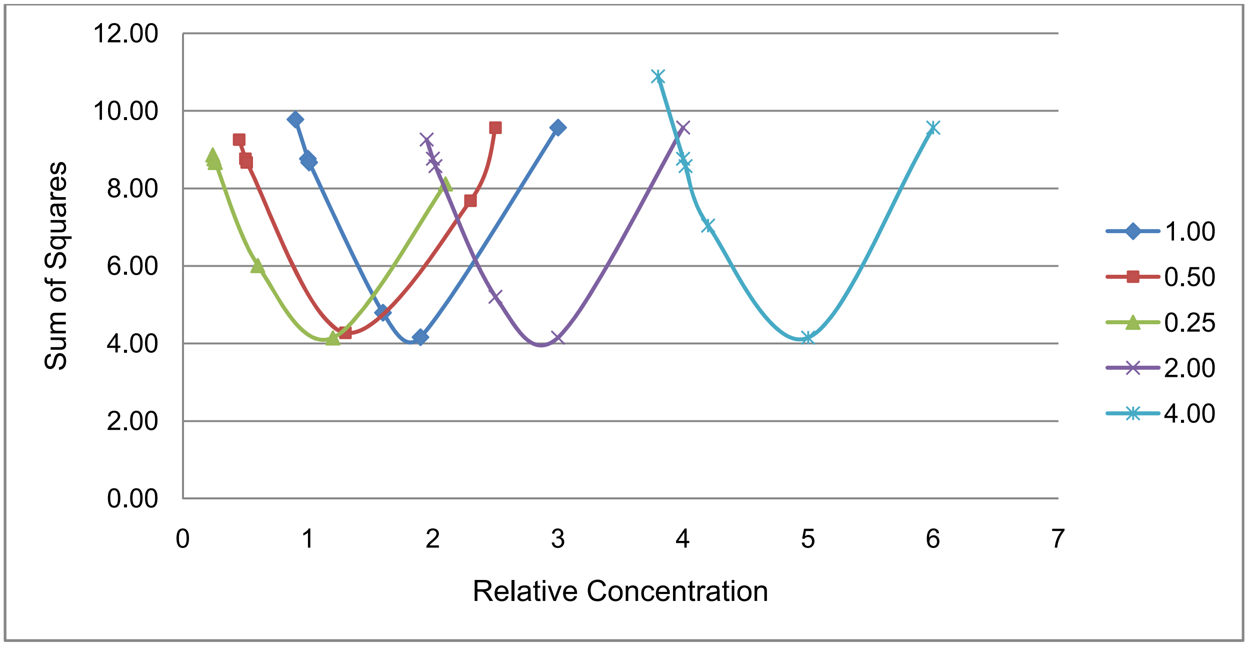

Figure 18. Plots of calculated minima of sum of squares (broad spectrum): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 of the base spectrum. The spectrum is broad and severely overlapped.

Table 25. Calculated concentration for component (I) in the two-component mixture with a severe degree of overlap (broad spectra): The relative concentration of component (II) was kept at 1.00

Expected Concentration of Component (I)	Concentration of Component (I) Found
1.00	1.90
0.50	1.30
0.25	1.20
2.00	3.00
4.00	5.00

Table 26. Calculated minima for component (I) in the two-component mixture with a severe degree of overlap (structured spectrum): The relative concentration of component (II) was kept at 1.00

1.00		0.50		0.25		2.00		4.00	
Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.	Rel. Conc.	Sum of Sq.
0.90	7.49	0.45	6.84	0.22	6.59	1.90	7.49	3.70	10.59
1.00	6.23	0.50	6.23	0.25	6.23	2.00	6.23	4.00	6.23
1.01	6.12	0.51	6.13	0.50	3.95	2.01	6.12	4.01	6.12
1.60	**2.84**	0.55	5.68	**0.70**	**3.02**	2.20	4.31	4.20	4.31
1.80	3.28	**1.10**	**2.84**	1.10	3.52	**2.70**	**2.96**	**4.50**	**2.91**
2.10	5.43	1.40	3.80	1.30	4.95	3.00	4.52	5.00	4.52
2.30	7.85	1.60	5.43	1.45	6.54	3.25	7.17	5.50	11.06

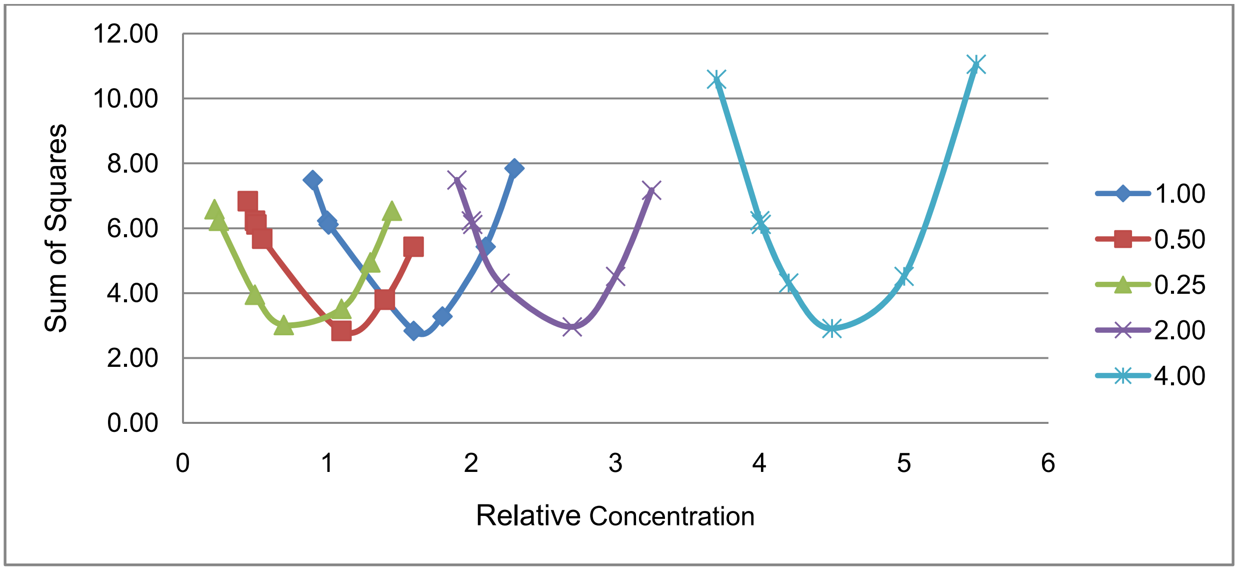

Figure 19. Plots of calculated minima of sum of squares (structured spectrum): The spectrum of component (II) is at a relative concentration of 1.00. The spectra of component (I) were shown with varying relative concentration of 0.25, 0.50, 1.00, 2.00, and 4.00 of the base spectrum. The spectrum is structured and severely overlapped.

Table 27. Calculated concentration for component (I) in the two-component mixture with a severe degree of overlap (structured spectrum): The relative concentration of component (II) was kept at 1.00

Expected Concentration of Component (I)	Concentration of Component (I) Found
1.00	1.60
0.50	1.10
0.25	0.70
2.00	2.70
4.00	4.50

CHAPTER 5

CONCLUSION

The concentrations of the individual components present in the simulated data as well as the experimental data were determined using the simultaneous equation method and the method of least-squares. A new spectral subtraction method similar to rank annihilation factor analysis was also attempted on simulated data.

Noise-free simulated data were used initially to observe the characteristics of the methods used for quantitation. The simultaneous equation method, by selecting appropriate wavelength units of the spectra gave, consistent results when the system was not too severely overlapping, and good calibration curves at the wavelengths chosen can be obtained. The method of least-squares analysis using the complete spectra gave consistent and reliable results when overlap was not severe. In situations where the spectral overlap is too severe, the least-squares method sometimes did not do as well as compared to the judicious selection of wavelength units in the simultaneous equation method.

The method of simultaneous equation was applied to the two component mixtures with broad spectra whose degree of overlap was varied with respect to their relative concentrations. Even in the situation where the spectral overlap was great, there were regions where the components absorbed alone. Hence, one could still find wavelength units where interference was minimal for use in analysis. However, the molar absorptivity of these regions tends to be small. Some choices of wavelength units selected gave somewhat less accurate results, but overall, the results obtained for noise-free simulated data were good. The least-squares analysis using the complete wavelength units of the spectra gave consistent and reliable results when overlap was not severe. In situations where the spectral overlap is severe the least-squares method in some instances depending on the choice of wavelength did not do as well as compared to the method of simultaneous equation. The severe degree of overlap did not affect the results at all in the case of noise-free simulated data.

Components with more structured features were also studied. It was found that the method of solving simultaneous equations and the method of least-squares were capable of giving good results in most instances for components with greater structural features and varying degree of overlap.

The findings were applied to experimental data of iron(III) and copper(II) azide complexes. Thus, for the experimental data the spectral range between 325 and 485 nm was used and this range included the significant absorbance peaks of the two components. The method of simultaneous equation was applied to quantitatively determine the concentrations of the individual component of iron(III) and copper(II) present in the varying volume ratio of iron(III) and copper(II) azide working solutions in the mixture. Mixtures with known concentration of the individual working standards solutions were prepared in the volume ratio; 1:1, 2:1, and 3:1 of iron(III) and copper(II) azide complexes and vice versa.

The simultaneous quantitative determination of the complexes in their mixtures using conventional spectrophotometric methods was hindered by unresolved peaks throughout the wavelength range selected, i.e. 325 to 500 nm.

The results of the method of simultaneous equations showed that the calculated concentrations of iron(III) and copper(II) azide complexes present in the mixtures deviated from the expected value and the percent error varied widely depending on the wavelength pairs selected for analysis. In this very challenging situation, the choice of wavelengths for analysis may not always help. This can be attributed to the fact that the regions of least interference are also regions where iron(III) and copper(II) azide complexes absorbs weakly and thus measurements errors were maximal.

The method of least-squares using the wavelength range 325 to 415 nm gave results that were an improvement as compared to the method of solving simultaneous equation. The percent error for the calculated concentrations of iron(III) and copper(II) in the mixture were in some mixtures, especially when the signals of the components were comparable (not too large or small), were within experimental error. This was quite good considering the extreme overlap. To seek further improvement in light of the inconsistent results obtained for the experimental data, a new total spectral subtraction method was tried. The results of the method were again good and accurate in the case of little overlapping spectral data but unsatisfactory when severe overlap occurs. The means of determining the “end point” of the subtraction remains elusive although several attempts had been made.

Future Direction

In the future, a real experimental data set with components that have stronger absorbers and with more structured spectra should be tried to see if better simultaneous quantitative results can be obtained.

Also a better means of quantitatively determining the correct subtraction for the spectral subtraction should be explored and a more automatic algorithm for the determination for the negative values after over subtraction has occurred should be written.

Thus this set of studies done in this research projects clearly points out the difficulties of simultaneous quantitative determination in complex mixtures. Much work by this lab and other workers are continuing and still needed.

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APPENDICES

APPENDIX A

Method of Simultaneous Equation

Table 1. Procedure for solving simultaneous equation for 1.0 mL iron(III) and 1.0 mL copper(II) azide solution using the Excel matrix operation.

	A	B	C	D	E	F	G
1	Method of Simultaneous Linear Equation With Excel Matrix Operations						
2	Wavelength	Molar Absorptivity, ε		Absorbance		Concentrations	
3	λ (nm)	$M^{-1}cm^{-1}$		of Unknown		in Mixture (M)	
4	330	1107	1420	0.177		2.85E-05	[Cu (II)]
5	400	3757	1015	0.211		1.02E-04	[Fe(III)]
6							
7	DOCUMENTATION						
8	Cell A4:A5=	Selected wavelengths					
9	Cell B4:C5=	Molar absorptivity of the selected wavelengths for Fe(III) and Cu(II)					
10	Cell D4:D5=	Measured absorbance of unknown at the selected wavelengths					
11	Cell F4:F5=	Highlight blank cells and, type the formula:					
12		[=MMULT(MINVERSE(B4:C5),D4:D5)]					
13		Press CONTROL + SHIFT + ENTER simultaneously on a PC					
14		Then the calculation is done and the answer shows up in cell F4 and F5					
15							
16							

APPENDIX B

Method of Least-Squares

Table 1. Procedure for the method of least-squares analysis for 1 mL iron(III) and 1 mL copper(II) azide solution using the Excel SOLVER.

	A	B	C	D	E	F	G	H
1	METHOD OF LEAST SQUARES ANALYSIS USING THE EXCEL SOLVER							
2				Measured	Molar			
3	Wavelengths	Abs. of Standard		Abs. of	Absorptivity, ε		Cal.	
4	λ (nm)			Mixture	$M^{-1}cm^{-1}$	$M^{-1}cm^{-1}$	Abs.	
5		Fe(III)	Cu (II)	A_m	Fe(III)	Cu (II)	A_{calc}	$[A_{calc}-A_m]$^2
6	325	0.095	0.046	0.195	1187.5	1150.0	0.192	1.001E-05
7	330	0.085	0.041	0.177	1062.5	1025.0	0.171	3.072E-05
8	335	0.082	0.044	0.174	1025.0	1100.0	0.171	1.149E-05
9	340	0.081	0.050	0.178	1012.5	1250.0	0.176	3.309E-06
10	345	0.082	0.057	0.185	1025.0	1425.0	0.186	6.831E-07
11	350	0.081	0.064	0.191	1012.5	1600.0	0.193	2.455E-06
12	355	0.081	0.071	0.197	1012.5	1775.0	0.201	1.414E-05
13	360	0.080	0.078	0.202	1000.0	1950.0	0.208	3.025E-05
14	365	0.079	0.083	0.205	987.5	2075.0	0.212	4.761E-05
15	370	0.078	0.087	0.209	975.0	2175.0	0.215	3.757E-05
16	375	0.077	0.091	0.211	962.5	2275.0	0.218	5.414E-05
17	380	0.075	0.094	0.213	937.5	2350.0	0.219	3.557E-05
18	385	0.073	0.095	0.214	912.5	2375.0	0.217	1.043E-05
19	390	0.071	0.096	0.214	887.5	2400.0	0.215	2.235E-06
20	395	0.069	0.096	0.213	862.5	2400.0	0.213	1.683E-07
21	400	0.067	0.094	0.211	837.5	2350.0	0.207	1.337E-05
22	405	0.064	0.092	0.207	800.0	2300.0	0.201	4.038E-05
23	410	0.062	0.088	0.203	775.0	2200.0	0.193	9.884E-05
24	415	0.059	0.084	0.198	737.5	2100.0	0.184	1.955E-04
25							sum=	6.389E-04
26	Standards	Concentrations in the mixture						
27	[Fe(III)] =	(to be found by solver)						
28	0.00008		[Fe(III)] =	1.16E-04				
29	[Cu (II)] =		[Cu (II)] =	4.68E-05				
30	0.00004							
31	Path length							
32	(cm) =							
33	1							
34								

Abs. = Absorbance, Cal. = Calculated

	A	B	C	D	E	F	G	H
35	DOCUMENTATION							
36	Cell A6:A24 =	Selected Wavelengths						
37	Cell B6:C24 =	Measured Absorbance of Each Standard at Selected						
38		Wavelengths						
39	Cell E6 =	B6/(A33*A28)						
40	Cell F6 =	C6/(A33*A30)						
41	Cell G6 =	E6*A33*D28+F6*A33*D29						
42	Cell H6 =	(G6-D6)^2						
43	Cell H25 =	SUM(H6:H24)						
44								

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