KINETIC STUDIES OF THE REACTION OF FORMALDEHYDE WITH PLASMA ALBUMIN IN WATER AND ETHANOL - WATER MIXTURES

BY

JOSEPH TORSHIAN UGYE

DEPARTMENT OF CHEMISTRY,
FACULTY OF SCIENCE,
AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA

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BY

Joseph Torhian UGYE B.Sc (UDUS 1996), MSc (ABU 2005)
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Declaration

I declare that the work in this thesis entitled "**Kinetic studies of the reaction of formaldehyde with Plasma albumin in water and ethanol - water mixtures**" has been performed by me in the Department of Chemistry under the supervision of Prof. A. Uzairu, Dr. S.O. Idris and Prof. H.O. Kwanashie. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

_____	_____	_____
Joseph Torshian Ugye	Signature	September, 2015

Certification

This thesis titled "**Kinetic Studies of the Reaction of Formaldehyde with Plasma Albumin in Water solution and Ethanol - Water mixtures"** by Joseph Torshian Ugye meets the regulation governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Prof. A. Uzairu Chairman, Supervisory Committee	Signature	Date
Dr. S.O. Idris Member, Supervisory Committee	Signature	Date
Prof. H.O. Kwanashie Member, Supervisory Committee	Signature	Date
Prof. V.O. Ajibola Head of Department,	Signature	Date
Prof. K. Lawal Dean, School of Postgraduate Studies	Signature	Date

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Dedication

This piece of work is dedicated to God Almighty for his infinite Grace, my grand parents Late Pa Ugye Afea, Late Konwua Ajunku and Aya. Elizabeth Konwua. My parents, Late Zaki Msar Ugye, and Mrs Mary Ngoban Msar for their vision for my training. The same also goes to my wife Mrs. Ugye Josephine and children especially Rev. Jacob Ugye for his support in preparing this document and all my friends who stood by me at every stage of this study.

Abstract

Formaldehyde is a simple member of the aldehyde family and one of the simplest organic molecules. It is known to be ubitiquious in nature in foods, domestic air, cigarette smoke etc and has been found to be toxic over certain doses. The chances of harmful effects are reported to increase under room temperature because of its volatility and have also been reported as occupational and environmental toxicant. In spite of these claims, there is paucity of knowledge of the reaction kinetics and mechanisms of this organic molecule with carrier proteins, common non-foods and food materials in the literature. In this study an investigation of formaldehyde levels in some selected foods, non-foods, cosmetics, human milk and urine samples based on spectrophotometric method of Hantzsch by formation of a derivative for separation was carried out using acetylacetone and acetoacetanilide reagents. A 3,5-diaphenyl-1,4-dihydropyridine adduct was also prepared from acetoacetanilide, formaldehyde and plasma albumin and was characterized by measuring its melting point, IR analysis, Uv spectra as well as the antimicrobial activity. The stoichiometry and the kinetic measurements were carried out at constant conditions with the concentrations of the formaldehyde in excess of 0.27 $x10^{-1}$ mol dm^{-3} while that of plasma albumin was kept constant at 0.51 $x10^{-4}$ mol dm^{-3}**.** The effect of dielectric constant, added ions , pH and ionic strength and acidity constant (pKa) on the rate of reaction were all monitored by varying their concentrations while keeping the concentration of the aqueous formaldehyde and all the other conditions constant The kinetic of the reaction of plasma albumin with formaldehyde in water and ethanol-water mixtures was also investigated using bronsted-type plot model. Both the acetylacetone and acetoacetanilide reagents methods found formaldehyde levels in the studied samples at a non significant amount with a ($p > 0.0001$) at 95% degree of confidence. Also the

prepared 3,5-diaphenyl-1, 4-dihydropyridine adduct was found to have a melting temperature range of 179.5 - 180.4^{o} indicating that it was a labile schiff base and a protein with glycol protein (lectin) properties. The stoichiometric data showed a mole ratio of 1:2 for formaldehyde to plasma albumin and the reaction was found to be pseudo-first order with respect to formaldehyde and overall second order at constant conditions in both systems. The rate of the reaction in water medium and ethanol water-mixtures was found to increase with increased pH, ionic strength and the added ions. The results of the activation parameters in water and ethanol-water mixtures showed a large negative entropy, small positive values of activation energy, negative values of enthalpies of reaction and a small positive Gibbs free energies. This shows that the reaction was not spontaneous and thermodynamically hindered or unfavourable but can take place by a collision or activated complex mechanism. The values of rate constants of the reaction between plasma albumin and formaldehyde were found to decrease with the increase in the concentration of ethanol and the reaction. The corresponding values of the Brønsted-type plots proportionality constants (β) for the reaction in water solution and ethanol-water mixtures were found to be $\beta = 0.059$ and 0.0021 respectively. The second order rate constant values of 0.099 ($\pm$0.034) $dm^3\ mol^{-1}\ s^{-1}$ in water and 0.06 ($\pm$0.028) $dm^3\ mol^{-1}s^{-1}$ ethanol-water mixtures respectively were obtained. Also appropriate mechanistic proposals were made for the reaction in water and ethanol-water systems. The proposals showed that one mole of formaldehyde binds two moles of plasma albumin forming Schiff bases slowly and reaction was found to operate by binding/ associative mechanism

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Abbreviations

ACSM:	Activated complex formed in the second mechanism
AGE	Advance glycosylation end - products
AFM	Atomic Force Microscopy
BAC:	Blood alcohol concentration
C_2H_5OH:	Ethanol
CIO:	Cosmetic information Organization
CTEA	Cosmetics, Toiletry and Fragrance Association
FTIR	Fourier Transform Infrared spectroscopy
$H_2C(OH)_2$:	Methylene glycol
HCHO:	Formaldehyde
$[H^+]$	Hydrogen ions concentration
HSA `	Human serum albumin
k_{obs} :	The observed second order rate constant in binary mixture solution.
$PABNH_2$:	Plasma albumin adduct
$PABNHCH_2OH$: `	Hydroxylmethylamino acid adduct
ORD	Optical rotator dispersion
OSHA	The Occupational Safety and Health Administration
US, DHHS	United States Department of Health and Human Services
SESMORTAC:	The study of effect of solvent mixture on the one-step reaction rates using the transition state theory and cage effect model.
Sg	Specific gravity
US, DHHS	United States Department of Health and Human Services

WHO	World Health Organization
WHAR3.10	World Health Assembly Resolution
$\Delta\varepsilon_{255}$	Change in absorbance at wavelength 255nm
$1/\Delta\varepsilon_{255}$	Reciprocal wavelength of 225nm
λ:	Wavelength of maximum absorption
χEtOH:	mole fraction of ethanol
χH_2O:	mole fraction of water

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CHAPTER ONE

1.0 INTRODUCTION

Formaldehyde is a colourless, flammable gas that has a distinct, pungent smell. It is a simple substance containing oxygen, hydrogen and carbon and the simplest member of compounds called aldehyde and a natural part of our atmosphere (WHO, 1989; IARC, 1995). Formaldehyde is a known gas at room temperature with a chemical formula CH_2O and it is found to be readily soluble in water. It is also reported to be widely present in aquatic and air environments. Relatively, it is considered to be an important organic compound in industrial synthesis as well as an indoor air contaminant (Khoder*et al*., 2002). It is reported widely to be present in aquatic and air environments (CEPA, 2004). Formaldehyde is also known as methanal, methyleneoxide, oxymythelene, methyl aldehyde, formalin, morbic acid, oxo methane and embalming fluid and is reported to occur naturally in foods (FDA 1998).

There are reported levels of natural formaldehyde in raw foods, ranging from 1 mg/kg up to 90 mg/kg and accidental contamination of food may occur through fumigation, the use of formaldehyde as a preservative, or through cooking (WHO,1989). Tobacco smoke as well as urea-formaldehyde foam insulation and formaldehyde-containing disinfectants are all known sources of indoor formaldehyde (CEPA, 2004). The major anthropogenic sources affecting humans are therefore reported to be from indoor environment (CEPA, 2004). Products containing formaldehyde, such as resins, glues, insulating materials, chipboard, plywood and fabrics, are common. (WHO, 2001).

The most commonly known commercially available forms are 30-40% aqueous solution (formalin), parasite-S, paracide-F and Formalin-F. These are usually used as formaldehyde releasing agents for the preservation of fish (FDA, 1998). Formaldehyde is known to be naturally produced in the human body in small amounts (ATSDR, 1999). It has been reported to occur in blood, different organs, fruits, seeds, micro-organisms,

plants and animals and is emitted as a by-product of certain vegetables, such as cabbage and Brussels sprouts when they are cooked (Goldernberg, 1994). It is also produced by high energy irradiation of carbohydrates or proteins and by autooxidation of unsaturated fat and fatty acids. It is known to be one of the volatile compounds formed in the early stages of the decomposition of plant residues in the soil (Goldernberg, 1994). Industrially, formaldehyde is used in the production of cosmetics and sugar, well drilling fluids, in agriculture as a preservative for grains and seed dressing. Other areas of its use include, the rubber industry, the production of latex, leather tanning, wood preservation and photographic film production in hospitals and laboratories to preserve corpse and tissue specimens (US - EPA, 1999).

Formaldehyde dissolves easily in water but does not last long enough in it. However, during this period aquatic life in water could come in contact with it before it is oxidized. Exposure to formaldehyde is reported to cause irritation of eyes, nose, throat and skin at low levels in humans while it is known to be carcinogenic at high levels (ATSDR, 2011). This organic substance has been found in at least 26 of the 1,467 of the United States national priorities list sites as identified by their Environmental Protection Agency. The Occupational Safety and Health Administration (OSHA) of the United States of America has set a permissible exposure limit for formaldehyde at 0.75mg/L for an 8hr working day or 40 hrs working week and the United States National Institute for Occupational Safety and Health (NIOSH) recommended exposure limit of 0.01mg/L (NIOSH, 1997). Families can reduce the risk of exposure to formaldehyde vapours by opening windows, the use of fans to bring in fresh air indoors, removing formaldehyde sources in the home, seal of unfinished manufactured wood surfaces and washing of new permanent press clothing before wearing can help lower exposure (ATSDR, 1999).

Microbial contamination of household products, especially those used around the eyes and on the skin, can cause significant problems and preservatives can help to prevent such problems of contamination by its anti microbial activity (Moennich *et al*., 2009).

A report from US Department of Health and Human Services (US, DHHS) showed that Formaldehyde and its releasing agents are widely used as preservatives in foods and most household products like, cosmetics, paint, cloth, baby underwear and toilet tissues, medicinal and industrial products in aqueous forms worldwide and has been reported to be first used as a biological preservative more than a century ago (US, DHHS, 2008; CIO, 2008). Formaldehyde-releasing preservatives are reported to be ingredients that are highly efficient in helping to ensure the safety of products by protecting them against contamination by micro organisms during storage and during continued use by consumers. They have the ability to release formaldehyde in very small amounts over time. The use of formaldehyde-releasing preservatives ensures that the actual level of free formaldehyde in the products is always very low but at the same time sufficient to ensure absence of microbial growth. If pure formaldehyde was used, addition of enough formaldehyde at the beginning to ensure preservation over the whole lifetime of a product would be necessary, because formaldehyde is slowly used up over time (CIO, 2008). Formalin which contains 37% w/v formaldehyde has been used as a therapeutant to control echo parasites and aquatic fungi disease event occurring at fish culture facilities. Commonly the fish are dipped in formaldehyde for this purpose. Its residue in food for human consumption are proscribed because it is a possible carcinogen (FDA, 2006). It is also known as a common solvent for tissue fixation and for corpse preservation as well as a bacteriostatic agent in some foods, such as cheese. Fruits and vegetables are found to typically contain 3–60 mg/kg, milk and milk products about 1

mg/kg, meat and fish 6–20 mg/kg and shellfish 1–100 mg/kg (Estani, 1992). Formaldehyde is a known toxin to humans. Its residue in food for human consumption are proscribed because of it's a possible carcinogen (Kiernan, 2000). The International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) therefore classified formaldehyde as a "probable human carcinogen" for a rare form of nasal cancer (IARC, 2004). The U.S. Environmental Protection Agency (US-EPA) has similarly classified formaldehyde as a probable human carcinogen under conditions of unusually high or prolonged exposure. Formaldehyde is not typically added directly to cosmetics and personal care products, other than as a component of some nail hardening products. Regarding inhalation of large amounts of formaldehyde, the IARC considered new studies again in 1995 and maintained the rating at 2A (probably carcinogenic to humans) (IARC, 1995). IARC also recommended a reclassification of formaldehyde as a known human carcinogen based on provision of sufficient evidence to establish that exposure to formaldehyde causes nasopharyngeal cancer in humans under certain circumstances. (IARC, 2004). The IARC also found strong evidence of a link between formaldehyde and leukemia, though the evidence was not sufficient to establish a causal relationship. Similarly, travel trailers and mobile homes provided by the United States Federal Emergency Management Agency (FEMA) between 2006-2007 for the displaced Gulf coast residents of Hurricane Katrina and Hurricane Rita victims were found to have average levels of $77 \mu g L^{-1}$ (FEMA, 2007). Exposure to these levels were reported to cause breathing difficulties, nose bleeds and persistent headaches to the residents. Long term exposure to levels in this range was reported to be linked with an increased risk of cancer and of respiratory illness. The levels detected were higher than those expected in indoor air where levels are commonly in the range of 10-20 $\mu g L^{-1}$ and were higher than those for Agency for Toxic Substances Disease Registry division of

the United States Canters for Disease Control and Prevention minimal risk level (ASTDR, 2006). Other reported exposures include cosmetic products containing formaldehyde. Formalin and/or Paraformaldehyde is found to come into contact with hair (e.g. shampoos and hair prep arations), skin (deodorants, bath products, skin preparations and lotions), eyes (mascara and eye make-up), oral mucosa (mouthwashes and breath fresheners), vaginal mucosa (vaginal deodorants) and nails (cuticle softeners and nail creams and lotions (Shimadzu,1973). Exposure from most of these sources was reported to be localized, although some formaldehyde was known to be available for inhalation (e.g. from shaving creams). Systemic absorption, including penetration into the circulatory system, is estimated to be negligible. There have been reports of newborn infants being exposed to formaldehyde containing disinfectants in incubators (Piletta-Zenin *et al.*, 2007).

The role of formaldehyde in mis-folding of neuronal tau protein, and the toxicity of formaldehyde-induced tau aggregates on human neuroblastoma cells has also been investigated (Chun *et al.* 2007). Formaldehyde, as a cross linking agent was found to react with thiol and amino groups, leading to protein polymerization (Yu *et al*., 2006). Furthermore, ethanol and methanol ingestion have been proved to be important public health concern because of the selective actions of their toxic metabolites namely formaldehyde and formic acid, on the retina, the optic nerves and the central nervous system (Yu, 2001). In the liver and retina, majority of methanol was found to be converted to formaldehyde, by alcohol dehydrogenase. Formaldehyde was then converted to formate by aldehyde dehydrogenase and other enzymes (Jacobsen and McMartin, 1997). In semicarbazide-sensitive amine oxidase (SSAO)-mediated pathogenesis of Alzheimer's disease, formaldehyde was shown to interact with β-

amyloids to produce irreversibly cross-linked neurotoxic amyloid-like complexes (Yu *et al*., 2006; Yu; 2001; Gubisne-Haberle *et al*., 2004). Other studies have also shown that formaldehyde exposure leads to formation of DNA/protein crosslink, a major mechanism of DNA damage. The DNA/protein cross links have been used as a measure of dose in drug delivery (WHO, 1989; Heck and Casanova, 1999). Formaldehyde is also known to be one of the products of incomplete combustion which is released to atmosphere (Yuan, 1985). In metropolitan cities, formaldehyde is reported to be the predominant aldehyde emitted by automobiles especially when alcohol-based fuels are used and also as a secondary product of photochemical reactions of volatile organic compound (Lei *et al.,* 2010).The main sources for the formaldehyde pollution in air are reported to include painting, coating material and cigarette smoking (US, EPA, 2012). However, formaldehyde is known to be toxic to humans and other animals at high concentrations and particularly toxic to humans at levels above 0.08% w/v of formaldehyde in human blood and irritating to the respiratory tract, eye and skin (Chun *et al.,* 2007). Inhalation of a large amount of formaldehyde is reported to cause severe nasal tumours and irritation of the upper respiratory tract in rats, mice and humans. Data from human exposures indicated that high concentrations of formaldehyde may lead to pulmonary edema (IARC, 2007).

The daily intake of formaldehyde has been reported to be difficult to evaluate, but a rough estimate from the available data is found in the range of 1.5 –14 mg/day for an average adult, most of it in a bound and unavailable form WHO, 1989, WHO, 2001). A sufficient amount of formaldehyde reaching target cells, and the saturation of formaldehyde metabolism to formate is reported to increase the covalent binding of formaldehyde to deoxyribonucleic acid (DNA). The workers were of the view that the

carcinogenicity of formaldehyde could result from its ability to induce DNA-protein cross-links or hydroxymethyl adducts in DNA (Fennel,1994). Formaldehyde has also been described as one of the chemical mediators of apoptosis, that is programmed cell death (Abubakar *et al.,* 2009). The presence of formaldehyde residue in the food meant for human consumption are therefore proscribed because of possible carcinogenicity (Jung *et al*., 2001). Moreover, the formaldehyde generated *in situ* from some N-methylated compounds have been reported to induce apoptosis or retardation of cell proliferation to tumorous cells known as cancer (Korpan *et al.,* 2000). ‘

Recent studies have shown that neuro degeneration is closely related to misfolding and aggregation of neuronal tau. Neuronal tau is an important protein in promoting and stabilizing the microtubule system involved in cellular transport and neuronal morphogenesis. The significant protein tau aggregation induced by formaldehyde and the severe toxicity of the aggregated tau to neural cells may suggest that toxicity of methanol and formaldehyde ingestion is related to tau misfolding and aggregation. Both formaldehyde and acetaldehyde can go through the blood-brain barrier and cause some lesions to CNS, especially our visual system. Formaldehyde is reported to penetrate through the blood-brain barrier and could cause some lesions to central nervous system, especially our visual system (Scherbakova *et al*., 1986). It has also been established that high levels of formaldehyde react with proteins and nitrogen atoms in the environment to form reversible and irreversible adducts in vitro and in vivo and clinically, the lethal dose of formaldehyde for human beings is found to be about 0.08% w/v in the circulation (Erkrath *et al.*,1981).

1.2 The Research Problem

Formaldehyde levels higher than those obtainable in typical indoor exposure levels of formaldehyde have been found in travel trailers and mobile homes used as emergency housing for displaced residents of Gulf Coast Region, United State of America by Hurricane Katrina and Hurricane Rita (FEMA, 2007; Harris *et al.*, 1981). Long-term exposure to Formaldehyde has been found to cause irreversible neurotoxicity implicated in cancer of the central nervous system (brain astrocytoma) (Kilburn, 1994, Stroup, *et al.*, 1986). In addition, inhalation of formaldehyde has been shown to cause behavioral and memory disorders in rats and has been classified as a probable neurotoxic agent (Pitten *et al.*, 2000). It has also been reported that inhaled formaldehyde gas has negative effects on the central nervous system, and these effects may appear acutely in the form of headaches, malaise, sleeping disorders, fatigue, anorexia and dizziness. Formaldehyde has been reported to have the potential of significantly mis-folding DNA and native soluble proteins into attendant amyloid fibrillation and insoluble fibrils comprising of cross- ß-sheets (Chun *et al.*, 2005; 2007). Formaldehyde has also been found to have the potential of oxidizing protein-adducts with the attendant increase in acid levels in the blood even if it remains in the human body for only a short time (Sipe *et al*.,2005). Formaldehyde is reported to be clinically toxic to humans at a concentration of 0.08 % w/v in the body (Erkrath, 1981). It has also been described as one of the chemical mediators that cause apoptosis (Abubakar *et al*., 2009). There are evidences to suggest that under physiological conditions glucose reacts with formaldehyde and other related oxidizing agents non-enzymatically with a wide variety of proteins to form glycated products. Non-enzymatic glycation of proteins which has been shown to be a potential problem during their storage in the food and biotech industries (Davis *et al.*, 2001) and is also implicated in human diseases such as diabetes (Al-Abed *et al.*, 1999; Stitt, 2001) is accelerated in the presence of formaldehyde.

Rajbar (1968) reported that human blood proteins like hemoglobin and serum albumin undergo a slow non enzymatic glycation with oxidizing agents such as formaldehyde, mainly by forming schiff bases between α - amino groups of lysine and some times arginine and glucose molecules in the blood. Elevated glycol albumin has been reported in diabetes mellitus (Iberg and Fluckiger, 1986). Glycation is found to result in the formation of advanced glycosylation end products (AGE) leading to abnormal biological effects. AGEs are reported to be antigenic and represent many of the important neo antigens found in cooked and stored foods and also found to be channel blockers for calcium, magnesium, etc (Vazquez *et al.*, 2008). Because of the toxicities of formaldehyde to humans, quest for information about how this substance causes specific disease conditions through its reactions with biomolecules in the human body has become a necessity. To date, there are a few scientific studies reporting the detailed mechanisms of the reaction of formaldehyde with bio-molecules, especially proteins.

In fact, Chun *et al*., (2007) were the only ones known to have reported the kinetics and mechanisms of the reaction of formaldehyde with a human protein, protein tau using a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method.Their study found that protein tau was crosslinked and aggregated by formaldehyde via a second order mechanism thus indicating that formaldehyde is neurotoxic. Formaldehyde in the human body is distributed by blood and its possible reaction with plasma albumin is worth exploring.

1.3 Justification of the Research

There has been an increase in the reported negative influences of formaldehyde to nature and humans which has necessitated that the routine analysis of formaldehyde in the environmental niches like water, fish, cosmetics, foods and non- food materials etc.

should be urgently carried out (Uzairu *et al.*, 2009). The role of formaldehyde in mis-folding of neuronal tau protein (Chun *et al*,2005) and the toxicity of formaldehyde-induced tau aggregates on human neuroblastoma cells has also been reported (Chun *et al.* 2007). Formaldehyde is reported to be a cross linking agent and is known to react with thiol and amino groups, leading to protein polymerization (Yu, 2001; Yu *et al*., 2006). Furthermore, ethanol and methanol ingestion has been proved to be an important public health concern because of the selective actions of their toxic metabolites, which include formaldehyde and formic acid, on the retina, the optic nerves and the central nervous system (Yu, 2001). In semicarbazide-sensitive amine oxidase (SSAO)-mediated pathogenesis of Alzheimer's disease, formaldehyde is shown to interact with β-amyloids to produce irreversibly cross-linked neurotoxic amyloid-like complexes (Yu, 2001; Yu *et al*., 2006; Gubisne-Haberle *et al*., 2004). It is therefore necessary to not only assess formaldehyde levels in foods, non-food products and body fluids but to kinetically investigate the interations of formaldehyde with other proteins such as plasma albumin in order to evaluate the health risks associated with its trace presence in the blood stream where plasma albumin protein is most abundant. In spite of the above known and documented toxicity of formaldehyde, there are only a few studies exploring its levels in some common foods, non-foods materials and some body fluids and the detail mechanisms of its interactions with the biomolecules in the literature. Furthermore most of the information available on formaldehyde reaction with proteins and biological tissues are only based on fixation reactions from tanning and embalmment industries where high concentrations and volumes of formaldehyde are applied to tissue sections at frozen conditions (Bedino, 2003). In fact to the best of our knowledge, there is no detailed study on the kinetics andmechanism of the reaction

between trace levels of formaldehyde with plasma albumin under normal and varying physiological conditions in water solution and binary ethanol-water mixtures or media.

Formaldehyde has also been clinically reported to be toxic to humans at a concentration of 0.08%w/v in the body (Erkrath, 1981) and has also been described as one of the chemical mediators that cause apoptosis (Abubakar *et al*.,2009). There are evidences to suggest that under physiological conditions glucose reacts with formaldehyde and other related oxidizing agents non-enzymatically with a wide variety of proteins to form glycated products. Non-enzymatic glycation of proteins which has been shown to be a potential problem during their storage in the food and biotech industries (Davis *et al.,* 2001) and is also implicated in human diseases such as diabetes (Al-Abed *et al.,* 1999; Stitt, 2001). The glycation reaction is found to be accelerated in the presence of oxidants such as reactive oxygen intermediates, free radicals, formaldehyde etc. Other studies have also shown that Formaldehyde exposure leads to formation of DNA/protein crosslink, a major mechanism of DNA damage. The DNA/protein cross links have been used as a measure of dose in drug delivery (WHO, 1989; Heck and Casanova, 1999).

The significance of the present study therefore is that an investigation of the kinetics and mechanism of the reaction of plasma albmin with formaldehyde in water solution and ethanol – water binary mixtures as well as an assessment of the levels of the latter in some consumable foods and non-food materials and body fluids will provide information on the possible sources of exposure to this toxin to humans. The study will also expose the health risk factors associated with its presence in the blood stream even at trace levels. Such information is expected to guide individuals, public health workers and health care providers as well as policy makers on health on health issues of the

dangers associated with elevated levels of formaldehyde and its reaction pathways when exposed to it in the environment.

1.4 The Research Hypotheses

This study was based on the following two hypothes (i) Null hypothesis, H: This states that formaldehyde is absent in commonly used food, non- food materials and human body fluids and that formaldehyde does not in anyway cross link native soluble proteins like plasma albumin hence does not constitute a human risk factor. (ii) The alternative hypothesis, H_1: This states that elevated values of formaldehyde are present in commonly used foods, nonfood materials and human body fluids and that formaldehyde cross links native soluble proteins like plasma albumin which may cause health hazards in humans.To verify which of the two hypotheses above holds, some selected foods, non-food products, human milk and urine samples were analyzed for formaldehyde. Also, the reaction of formaldehyde with protein albumin in water and various ethanol- water mixtures was studied kinetically under various conditions similar to human blood serum in order to explore the health risks associated with the presence of formaldehyde levels in the human blood.

1.5 Aim and Objectives of the Study

The aim of this investigation was to measure formaldehyde levels in some foods,
Non food materials and body fluids as well as study the kinetics and mechanisms of the reaction of formaldehyde with plasma albumin in water and ethanol-water mixtures under various conditions similar to human blood. This was hoped to be achieved through the following objectives:

(i). To detect and measure amounts of formaldehyde in commonly used foods,non-food materials some body fluids and using acetylacetone and Acetoacetanilide reagents.

(ii). To isolate, purify, fractionate and characterize plasma albumin from whole blood samples using ethanol a common organic solvent with the view to studying its reaction with formaldehyde.

(iii).To investigate the kinetics of the reaction of formaldehyde with plasma albumin under normal and physiological conditions involving pH, temperature, reactants concentrations and ionic strength in water and (0 - 25%) ethanol - water mixtures.

(iv). To propose plausible mechanism(s) for the reactions of formaldehyde and plasma albumin using various kinetic models like Bronsted - plot and rate equations.

CHPTER TWO

2.0 LITERATURE REVIEW

2.1 Formaldehyde

Formaldehyde has a chemical formula of CH_2O and is the most simple known aldehyde. It is a colourless gas with a boiling point of -21^0C and readily polymerizes at room temperature and pressure and has a relative molecular mass of 30.03 g/mol with a pungent odour. It is found to have a dipole moment of 2.33D which makes it highly soluble in water. Formaldehyde is also known as formalin, embalming fluid, or formol. The term formalin refers to the aqueous solutions. It can be obtained from its cyclic trimer trioxane and polymer paraformaldehyde and exist mostly in water as the hydrate, $H_2C(OH)_2$. .Formalin is known to also occur in solid state as polymers (paraformaldehyde and trioxymethylene).The aqueous solution of formaldehyde usually

referred to as formalin (100% w/v) consists of formaldehyde of about 40 v/v or 37% w/v in water with small amount of stabilizer usually methanol in addition to limit its oxidation and polymerization reactions. A typical commercial grade formalin may contain 10-12% percent methanol in addition to metallic impurities such as aluminium (3 mg/L), iron (1mg/L) and copper(1mg/L) (Bedino, 2003).

2.2 Synthesis and Industrial Production of Formaldehyde

Formaldehyde was discovered in 1867 by a German Chemist, August Wilhem Von Hofman, but could not isolate it in its pure form during his life time. In 1892, the year Von Hofman died, another famous German Chemist Friedrich, August Kekule Von Stradanitz isolated formaldehyde in its pure form. Formaldehyde is produced industrially by the catalytic oxidation of methanol. The most common catalysts are silver metal or a mixture of an iron and molybdenum or Vanadium oxides. In the more commonly Formox process, methanol and oxygen react at about 250 - 400 ^{0}C in the presence of iron oxide in combination with molybdenum according to the chemical equation

$$2CH_3OH + O_2 \rightarrow 2CH_2O + 2H_2O \quad \text{---------- 2.1}$$

Formox is a world wide registered trade mark owned by Perstrop Speciality Chemicals, AB,Sweden. It is a high performance process which produces formaldehyde concentration of above 55% with minimum usage of methanol (Anonymous, 1963). The silver – based catalyst usually operates at higher temperature , about 650 ^{0}C as given in the reaction below.

$$CH_3OH \rightarrow H_2CO + H_2 \quad \text{---------- 2.2}$$

In principle, formaldehyde could be generated by oxidation of methane, but this route is not industrially viable because methanol is more easily oxidized than methane.

The annual world production of formaldehyde in 1995 was reported at 21 metric tonnes (US EPA, 1999).

2.3 Sources of Formaldehyde

The oxidation of organic materials in human and some fungi and algae like Candida is reported to produce formaldehyde and its homologous members. Formaldehyde is found to be a common environmental risk factor in paint, cloth, cigarette, exhaust gas and many other medicinal and industrial products (Quievryn and Zhitkovich, 2000). A recent study report in Denmark has revealed that one third of all cosmetics products and baby toilet moistened tissues contained formaldehyde or formaldehyde releasing preservatives (Piletta-Zenin *et al.,* 2007). It has also been observed that formaldehyde gas present in workrooms at concentration ranges as low as 1 to 11 mg/L can cause eye, nose, and throat irritation. Formaldehyde has been found not only as a potential carcinogenic chemical, but also it has become a matter of concern due to sick house syndrome in Japan due to formaldehyde concentration levels in domestic air in newly constructed houses which were reported to vary from ambient level (1-25 ppb) to as high as 4ppm (Sritharathikhun *et al*., 2004). The Cosmetics Ingredient Review Expert panel (1984) assessed the safety of formaldehyde and concluded that the formaldehyde levels of cosmetic products applied to the human skin should not exceed 0.2%w/v. Formaldehyde in high concentration is however found to be used as the wet strength resins added to sanitary paper products such as baby pampers, table napkins, facial tissues, pads and roll towels (IARC, 1995).

2.4 Uses of Formaldehyde

Formaldehyde is used as a chemical intermediate in the synthesis of other chemicals when reacted with phenols, urea or melanine to produce hard thermoset phenol formaldehyde resin, urea formaldehyde resin or melanine formaldehyde resin respectively. These resins are reported to be commonly used in permanent adhesives such as those used in plywood or carpeting. Formaldehyde is also known to be used as an analytical reagent in concrete, plaster, additives, cosmetics, disinfectants, fumigants, photography, biocide, reducing agent, corrosion inhibitor in oil wells and industrial sterilant. It is also used in variety of industries such as medical, detergent, food, fertilizer, wood petroleum etc. Often, formaldehyde is added to cosmetics to act as a preservative and an antimicrobial agent, and in agriculture as fumigant, germicide, fungicide and insecticide (Goldernberg, 1994). Formaldehyde is used as the wet strength resins added to sanitary paper products such as baby pampers, table napkins, facial tissues, pads and roll towels. It is reported to be combined with methanol and buffer to make embalming fluid. It is also used in the manufacture of acetal resins, 1,4-butanediol, hexamethylenetetramine, urea-formaldehyde, methylene diisocyanate, ethyleneglycol, and a variety of other chemicals (US EPA,1999) . Many other applications of formaldehyde in scientific processes are reported. It has also been reported as a contrast enhancer in gram's staining analysis (Uzairu *et al.*, 2010).

2.5 Toxicity of Formaldehyde

There has been renewed interest on the toxicity profile of formaldehyde, particularly the WHO and other toxic materials monitoring bodies like ATSDR which updated their profiles on formaldehyde up to 2012. ATSDR (2012) reported that formaldehyde affects organ systems like dermal (skin), gastrointestinal (digestive), immunological (immune

system), respiratory (from the nose to the lung systems and is known to be a human carcinogen.

2.5.1 Case reports and clinical studies on formaldehyde

Formaldehyde exposure has been reported to lead to formation of DNA-Protein cross link, a major mechanism of DNA damage. The DNA-Protein adduct formation has been used as a measure of dose in drug delivery (US EPA,1999). Formaldehyde as a cross linking agent is known to react with thio and amino groups leading to protein polymerization (Yu, 2001). There are reported cases of irritation of the eyes, nose, and throat by volunteers who were exposed to formaldehyde within the range of 0.25-3.0 mg/L (WHO, 1989). Formaldehyde is found to be extremely dangerous to life and health at 20mg/L.Severe pain, vomiting, chest pain, coma and possibly death are reported to occur when exposed to formaldehyde in extreme cases (ATSDR, 2012). Exposure could also result in corrosion of the gastrointestinal tract, inflammation and ulceration of the mouth, oesophagus and stomach (WHO, 1989). It is also known to cause menstrual disorders in female but cases like birth defect have not been reported. It is also an irritant and can trigger asthma allergies and cause particularly myeloid leukaemia in human (US, EPA 2012).

2.5.2 Formaldehyde as a risk factor for cancer

Based on limited evidence in human and sufficient evidence in animals, an increased incidence of nasal squamous cell carcinomas was observed in long term inhalation studies in laboratory rat and mice, implicating formaldehyde as a probable carcinogen to humans (ASTDR, 2011). These studies have suggested that formaldehyde exposure can cause cancer of the lungs and respiratory tracks in humans (ASTDR, 2011). The risk of

cancer is found to depend on the exposure level, duration and sensitivity of individuals. However the rate of lung cancer death was found to decrease with higher levels of formaldehyde exposure (Goldernberg, 1994). This observation lead researchers to conclude that factors other than formaldehyde exposure may have caused the increased death. Formaldehyde and cancer of various organs have been examined extensively in epidemiological studies (AOAC, 1980). In occupationally exposed populations, there were over 30 cohorts and case-control studies of professionals to check the risk of formaldehyde exposure. Exposure to formaldehyde vapours was found to cause alteration of tissue protein and this was reported to lead to local toxicity and promote allergic reaction. It was also found to reduce body temperature, loss of vision from direct splashing from solution containing formaldehyde. It could also lead to dead pulmonary edema with residual cardiac impairment (AOAC, 1980).

In a survey of 57 embalmers who were exposed to formaldehyde below 2mg/L, it was discovered that 81% of them complained of irritation effect on the eye, 75% complained of nose and throat, 33% cough, 23% chest pain,12%, wheezing and 11% shortness of breath (AOAC, 1980). Potential occupational exposures to formaldehyde were classified as follows: Agricultural workers, anatomists, beauticians, biologists, book binders, botanists, chemical production workers, cosmetic formulators, grease resistant textile finishers, disinfectant makers, disinfectors, dress-goods shop personnel, electrical insulation makers, glue and adhesive makers, ink makers, histology makers, mirror manufactures, paper makers, photographic film makers, plastic workers, textile mordanters and printers (AOAC, 1980). The International Agency for Research on Cancer (IARC) after an evaluation concluded that formaldehyde is a “probable human carcinogen” and it was reclassified in June, 2004, after a re-evaluation of all the existing data (IARC, 1995). The presence of formaldehyde residue in the food meant for human

consumption is therefore proscribed because of possible carcinogenicity (Jung *et al.*, 2001).

2.5.3 Laws and actions of some countries on the use of formaldehyde

The California Air Resources Board established new regulations in April, 2007, to regulate formaldehyde emissions from composite wood products including particle board, medium density fibre board and interior plywood. Europe and Japan have also established strong standards to reduce or virtually eliminate the use of formaldehyde and its emission into buildings from some of the building materials that emit formaldehyde into homes. A two steps process was set as limits on emissions for products manufactured after January, 2009. These standards although would not eliminate the addition of formaldehyde during product manufacture, but will make formaldehyde free alternatives much more competitive (FEMA, 2007).

In Europe, formaldehyde/formalin production, sales and marketing was banned in September, 2007 after nearly 10 years of planning.This ban on sales and marketing came on the heel of a ban on phenol in 2006. The ban meant that anyone currently using formaldehyde based products for embalming would need to seek an alternative or risk prosecution (FEMA, 2007). In the United States of America,agencies like Occupational Safety and Health Administration (OSHA), the Food and Drug Administration (FDA), Agency for Toxic Substances and Disease Registry (ATSDR) and Environmental Protection Agency (EPA) etc are charged with the responsibilities to provide valuable guidelines to protect public health. OSHA has set a permissible formaldehyde exposure limit of 0.75mg/L for an 8-hr work day and 40-hr work week. The National Institute for

Occupational Safety and Health (NIOSH) recommends exposure limit of 0.016 mg/L and a 15-minutes ceiling limit of 0.1mg/L ((NIOSH, 1997).

In Sweden and Japan, formaldehyde has been banned from cosmetics and toiletries by Cosmetics, Toiletry and Fragrance Association (CTEA). Australia and New Zealand have no regulation on formaldehyde while the southern Asia, Singapore, Malaysia, India and Hong Kong still use formalin in cosmetics. Volatile toxic aldehydes especially formaldehyde have recently been classified by the International Agency for Research on Cancer (IARC) in the Group 1 as carcinogenic to humans (IARC, 2004). The regulated value in domestic air for formaldehyde now in Japan is reported to be 100μg m^{-3} (Cosmetic Ingredient Review, 1984).

2.6 Methods used for the Determination of Formaldehyde

Various methods for the determination of formaldehyde have been proposed, out of these, spectrophotometric methods have been widely used (Li *et al., 2007).* These spectrophotometric methods are based on organic or inorganic reagents such as Schiff's reagent, p-phenylenediamine, chromatropic acid, p-amino ozobenzene, brilliant cresol blue-bromate, etc. Other modern methods such as fluorometric method, HPLC using 2-4-dinitrophenylhydrazine as a derivating agent are also reported (Li *et al., 2007).* However, most of these spectrophotometric methods are not sensitive enough and can be subjected to numerous interference from co-existing substances and also require long analysis time. The spectrophotometric method based on the Hantzsch reaction which involves the cyclization of amine, aldehyde and acetylacetone, a ß- diketone to form a yellow product of 3,5-diacetyl,1,4 -dihydrolutidine derivative at up to 40 ^{0}C has been reported to be sensitive, simple for formaldehyde determination in solid and aqueous

solution. This method is also found to be rapid and could be applied even on biological samples like proteins at low temperatures without destruction and denaturing of the samples (Li *et al.*, 2007). Formaldehyde can be extracted from foods using organic solvents such as ethanol, isopentane or by steam distillation and extraction with ether etc. The food stuffs may be homogenized before extraction (Hurley, 1994).

2.7 Blood Plasma Albumin

Blood is known to be the medium in which dissolved gases, nutrients, hormones and waste products are transported. Blood along with the heart and the blood vessels (e.g. veins and arteries) are reported to comprise the circulatory system of the body. The circulatory system helps in maintaining balanced conditions within the body i.e. homeostasis within a pH range of 7.3 -7.5. Blood is found to compose of straw-coloured liquid called plasma which contains suspended cells. The different specialized cells found in blood are red blood cells, white blood cells and platelets. Approximately 90% of plasma is found to be water- blood's solvent with the rest composed of dissolved substances, primarily proteins (e.g. albumin, globulin, and fibrinogen). Plasma is found to typically account for 55% w/v of blood and of the remaining 45% w/v of the greatest contribution is from the red blood cells (Helmenstine, 2007) as presented in Table 2.1

Human serum albumin is the most abundant protein in human blood plasma. It is produced in the liver. Albumin constitutes about half of the blood serum protein. It is soluble and monomeric. Albumin transports hormones, fatty acids, and other compounds, buffers pH, and maintains osmotic pressure, among other functions. Albumin is synthesized in the liver as pre pro albumin, which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum. The product, pro albumin, is in turn cleaved in the Golgi vesicles to produce the secreted albumin.

Table 2: 1 Composition of blood plasma.

Components	Percentage
Water	~92
Proteins	6-8
Salts	0.8
Lipids	0.6
Glucose (blood sugar)	0.1

The reference range for albumin concentrations in blood is 3.4 to 5.4 g/dL (Sugio *et al.*, 1999). The gene for albumin is located on chromosome 4 and mutations in this gene can result in anomalous proteins. The human albumin gene is 16,961 nucleotides long from the putative 'cap' site to the first poly (A) addition site. It is split into 15 exons that are symmetrically placed within the 3 domains thought to

have arisen by triplication of a single primordial domain. It has a serum half-life of approximately 20 days. The human isoform of serum albumin is found to be a 67 kD protein with 585 amino acids, of which 35 are cysteine and cystines. Only the first cysteine in the sequence is known to remain a free thiol while the rest participate in the formation of 17 disulfide bonds; these characteristics of the cysteine residues are conserved in the serum albumin of all vertebrates (Peters, 1996).

2.8 Plasma Albumin Chemistry

Plasma Albumin hereafter referred to as ($PABNH_2$) is generally regarded as serum albumin. It is known to be the smallest and most abundant of the plasma protein constituents with a molecular weight of 66300 to 69000 and an isoelectric point at pH 4.7 lower than that of the other proteins (White *et al.*, 1968). Human and Bovine albumin are found to be very similar in terms of structure and physicochemical properties. A human albumin molecule is reported to consist of a single and quite long compactly folded polypeptide chain of about 585 amino acid residues in length. It also has an almost spherical configuration with seventeen disulphide bonds with one Aspartic amino acid terminal group and six amino acid residues comprising of Glutamic acid, Alanine, Histidine, Lysine, Serine and Glutamic acids as side chains (White *et al.*, 1968, Tanaka *et al., 2001*)

Plasma albumin is known to be very soluble in water and the most abundant protein in the circulatory system, about 5g/100cm^3 of human blood volume and contributes 80% to colloid osmotic blood pressure (Sugio *et al.*, 1999., Carter and Ho, 1994). It has been established that serum albumin is chiefly responsible for the maintenance of blood. In mammals, albumin is reported to be synthesized initially as pro albumin by the liver, after removal of the signal peptide, the resultant pro albumin is further processed by removal of the six residue pro peptide from the new N-terminals.

2.8.1 Structure of serum albumin

Human serum albumin (HSA) is a known carrier for several drugs and by this it plays an important role in the regulation of plasma concentrations of these, including both endogenous and exogenous compounds. HSA is reported to share 76% homology with Bovine serum albumin (BSA) and is easily accessible, of low

cost and of medical importance and is the most abundant protein found in plasma. Several studies have revealed that HSA has high affinity to a very wide range of materials, including, metals such as Cu^{2+} and Zn^{2+}, fatty acids, amino acids, metabolites such as Bilibrubin and for many drug compounds (Waldmann,1977). The most important physicological role of the protein is therefore thought to be to bring such solutes in the blood stream to their target organs as well as to maintain the pH and osmotic pressure of plasma. In addition to its ordinary clinical applications such as hypovolemic shock treatment, many investigators have attempted to ultilize HSA as a carrier to deliver various drugs to their specific targets (Sugio *et al.*, 1999., Feshke *et al.*, 1981). The human plasma albumin structure is as presented (Appendices A and B).

The primary sequence of HSA shows that the protein is a single polypeptide with 585 residues containing seventeen pairs of disulphide bridges and one free cystein (Dugiaczyk *et al.*, 1982). Human serum albumin as well as serum from other species have been found to consist of three homologous helices domains (I–III). Each domain contains ten helices which are divided into anti-parallel six-helices and four sub domains. HSA contains 585 amino acid residues with only one tryptophan located at position 214 along the chain, probably derived through gene multiplication (Brown, 1976). The gene for albumin is said to be located on chromosome 4 and mutations in this gene can result in anomalous proteins. The human albumin gene is 16,961 nucleotides long from the putative 'cap' site to the first poly (A) addition site. It is split into 15 exons that are symmetrically placed within the 3 domains thought to have arisen by triplication of a single primordial domain. It has a serum half-life of approximately 20 days. The human isoform of serum albumin is a 67 kilodaltons (kD) protein with 585 amino acids, of which 35 are cysteine and cystines. Serum albumin is used in the clinical setting for the treatment of hypovolemic shock, severe burns, and many other medical conditions. Its commercial value exceeds one billion dollars annually (Peters, 1996). Out of the many functions served by serum albumin, its ability to bind and transport long chain fatty acids is particularly important and is essential for the effective metabolism of these highly insoluble molecules (Peters, 1996). Keaney *et al.*, (1993) suggested that serum albumin can carry nitric oxide as an S-nitroso adduct and also proposed that serum albumin has the properties of endothelium-derived

relaxing factor. In mammals albumin is reported to be synthesized initially as pro albumin by the liver, after removal of the signal peptide, the resultant pro albumin is further processed by removal of the six residue propeptide from the new N-terminus. Human serum albumin (HAS) is a known carrier for several drugs and by this it plays an important role in the regulation of plasma concentrations of these, including both endogenous and exogenous compounds.. Albumin transports hormones, fatty acids, and other compounds, buffers pH, and maintains osmotic pressure, among other functions. Albumin is synthesized in the liver as pro albumin, which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum.

2.9 Isolation and Purification of Plasma Albumin from Human Blood Samples using Organic Solvents

It has been known for some time that trichloroacetic acid precipitates of certain proteins dissolve in methanol whereas those of other proteins do not (Michael, 1968). Several authors (Korner and Debro, 1956., Schwert, 1957, Jarnefelt, 1961) have previously investigated the properties of albumin after treatment with trichloroacetic acid and alcohol or acetone .They have arrived at the conclusion that the albumin, after recovery was still in the native state, as judged by its physicochemical properties and by its unchanged antigenic properties but that it has acquired a slightly changed susceptibility towards proteolytic enzymes (Ehrenpreis *et al.,* 1958**).** A more detailed study of the behaviour of the main blood proteins and some other proteins have now shown that the solubilising action in suitable organic solvents, on albumin, but not on globulins, is shared by a number of other acids (Michael, 1968). Some acids were found to be unable to prevent damage to the albumin in the organic solvents, whereas others preserved the albumin in a condition that is more nearly related to the native state. This has enabled a process of separation of blood proteins to be developed which gives albumin of a high grade of purity, in high yields. In the study conducted by Michael, methanol and 0.1 molar HCl were used to investigate the properties of albumin obtained from human

serum. Solutions of those albumin specimens which had been made with methanol or ethanol as solvent and hydrochloric, formic or trichloroacetic acid, or with acetone in combination with trichloroacetic acid, did not show any opalescence or turbidity at any pH. On the other hand, solutions of albumin that had been prepared by using one of the other acids yielded turbidities or precipitates at or near pH 5. This was taken to indicate that such solvent systems denature the albumin to a certain degree. It must be assumed that in the less successful solvent systems the albumin is denatured to such an extent that it is not completely soluble and that therefore the subsequent recovery of albumin is smaller (Michael, 1968). This present study therefore employed ethanol and 0.14% HCl acid to undertake the isolation and fractionation of whole human blood with the hope to obtain native state and high purity plasma albumin and as a way of exposing the efficacy of ethanol as another organic solvent that could be used to isolate plasma albumin.

2.10 Formaldehyde Stabilizers

Formaldehyde solutions are unstable and degrade if not stabilized. The most popular stabilizer is methanol at usually 3 - 8% w/v in solution. Methanol rapidly forms a methylal with formaldehyde in water and is strongly favoured as a reaction product. The stabilization results from an inhibition of cross-reaction and cross-addition to polymeric forms. By stabilization, up to 30% of formalin can exist as the monohydrate methylene glycol. Methanol is chosen because of its availability and it is a natural by - product of manufacture of formaldehyde. It is possible to utilize other stabilizers such as ethanol or even glycols or glycerine. Other curious polymeric forms of formaldehyde exist and can be isolated, one example being trioxane, a cyclic polymer. Over time, during storage, formalin solutions degrade by natural oxidation changes, caused by air or photo oxidative pathways. Acidification increases, usually reaching a pH of 4 or less

with the production of formic acid and formate ions. This is the reason why the stabilization of formalin solutions is critical. Typically, formaldehyde reactivity and polymerization increases as acidity increases, particularly at pH of 6.5 and less. Basicity however substantially inactivates and slows formaldehyde reactions at a pH of 8-9 and essentially becomes non-reactive above pH of 9 (Bedino, 2003).

2.11 Reaction Between Proteins and Formaldehyde

The potential of formaldehyde to induce protein tau misfolding leading to neurodegenerative disease has been investigated and it was observed that aqueous formaldehyde at low concentrations induces tau protein to form amyloid-like aggregates (Chun *et al.,* 2007). A Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) experimental result by the workers showed tau polymerizing in the presence of formaldehyde, although under the same experimental conditions, polymerization of bovine serum albumin (BSA) or α-synuclein was not markedly detected. A further kinetic study by the workers shows that tau significantly misfolds and polymerizes in 60 minutes in 0.1% formaldehyde solution. A study of insulin reaction with excess formaldehyde (CH_2O) and deuterated formaldehyde (CD_2O) has reported that insulin was modified by formaldehyde and deuterated formaldehyde (Metz, 2006). Another study by Yu *et al.,* (2006) has also reported that semicarbizide-sensitive amine oxidase (SSAO) catalyzes the conversion of methylamine to formaldehyde. In Semicarbazide-sensitive amine oxidase (SSAO)-mediated pathogenesis of Alzheimer's disease, formaldehyde is reported to interact with β-amyloid and produces irreversibly cross-linked neurotoxic amyloid-like complexes. Therefore, the potential effect of formaldehyde on protein mis-folding is predicted to be

significant, even if formaldehyde remains in the human body for only a short time. Semicarbazide- sensitive amine oxidase (SSAO) is also reported to circulate in blood. There are also reported cases of increase in Serum SSAO activity in patients with a variety of vascular disorders, such as diabetes, multiple types of cerebral infarction and congestive heart failure. High blood SSAO has been identified to be a potential prognostic risk factor of mortality for the heart patients (Boomsma *et al.*, 2000). There are strong correlations of formaldehyde as a risk factor for the above physiological and pathological disorders which contributes to protein deposition, the formation of plaques and inflammations etc. A sufficient amount of formaldehyde reaching target cells, and the saturation of formaldehyde metabolism to formate is found to increase the covalent binding of formaldehyde to deoxyribonucleic acid (DNA) and the carcinogenicity of formaldehyde and could result from its ability to induce DNA-protein cross-links and/or hydroxyl methyl adducts in DNA (Fennel, 1994).

Formaldehyde is found to cross link, inactivate, stabilize, or immobilize proteins. It has been reported to react with the amino groups of the N-terminal amino acid residues and the side chains of argine, cystein, histidine, and lysine residues when treated with proteins (Bedino, 2003). Other investigations have found that under mild conditions formaldehyde irreversibly combine with the basic groups of proteins forming cross-links between the following pairs of groups: amine and amide; amine and phenol; amine and indole. Formaldehyde cannot, in their opinion, form a stable link between two amine or two amide group. The formaldehyde treatment has been found to result in a large variety of chemical modifications in proteins, such as the formation of methylol groups, Schiff bases and methylene bridges. Depending on the peptide sequence, methylol groups, Schiff bases, and methylene bridges are reported to be formed. The

most important modification of proteins induced by formaldehyde is reported to be the formation of stable methylene bridges (Bedino, 2003),. Furthermore a molecule of water and a methylene bridge forms as presented in Figure 2.2.

Reactions of higher values of aqueous formaldehyde with proteins are reported to be based on classical carbonyl-amine reaction chemistry. Amines and related nucleophiles are found to react with aqueous formaldehyde to form various intermediates with ultimately methylene bridging (-CH_2-) resulting in fixation or tanning type action. Primary amines are known to react to form intermediate hydroxymethyl groups that drives a basicity loss with pKa drops of about 4-5 units. Subsequently, but slowly, dehydration or condensation reaction occurs by loss of nwater molecules. Also possibly, after initial reactions are dimethylene ether linkages and the reduction of the hydroxymethyl groups by formaldehyde itself to methyl groups with production of formic acid as an endpoint product. These reducing properties of formaldehyde are reported to be accelerated in alkaline conditions where formaldehyde is known to precipitate the metals of various salts, such as bismuth, copper and silver (Bedino, 2003).

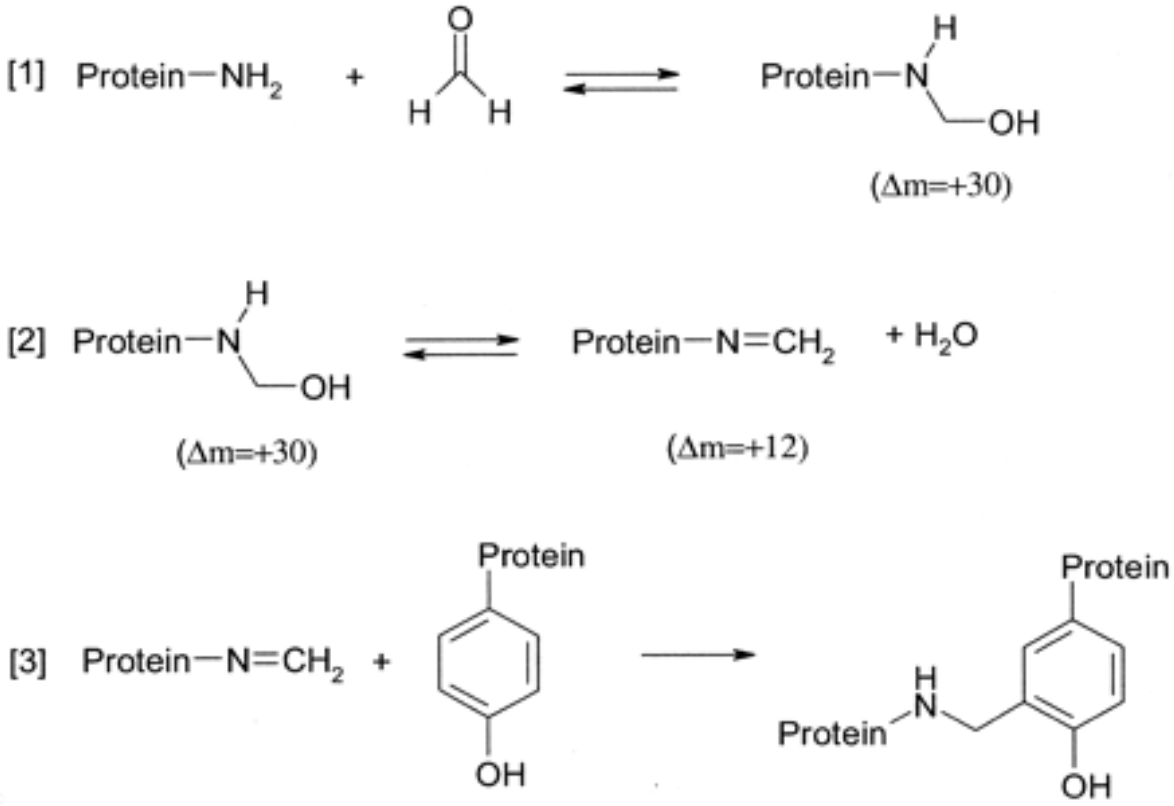


Figure 2.2: A variety of chemical modifications of formaldehyde in proteins (Bedino, 2003).

[1]. Formation of methylol groups [2].Formation of Schiff bases [3].Formation of methylene bridges.

2.12 Reaction of Proteins Tau with Formaldehyde

One of the known studies that reported the reaction of formaldehyde with proteins is the one by Chun *et al.,(*2007) which investigate the kinetics of tau aggregation in the presence of low-concentration formaldehyde using an atomic force microscopy (AFM). Neuronal protein tau was incubated in 0.1% formaldehyde and aliquots were taken for AFM observation at different time intervals. . The results showed a rapid start (within 10 min) of aggregation in the presence of formaldehyde and a remarkable increase of particle size in about 60–120 min. Parallel SDS-PAGE measurements also revealed a similar time evolution. The kinetic change in the size of aggregates showed a rapid polymerization of tau during the first 60 min. Analysis of the polymerizing kinetics was performed using Tsou's method (Tsou, 1988).

Tsou showed a linear procedure for tau polymerization in the presence of formaldehyde with a first order rate constant of 2.6×10^{-4} s^{-1}. As a control, no aggregation was detected by SDS-PAGE in the same time course when acetaldehyde, instead of formaldehyde, was added to the tau solution. Recent studies have shown that Neuro degeneration is closely related to misfolding and aggregation of neuronal tau. Neuronal tau misfolds and aggregates into globular-like polymers in 0.01– 0.1% formaldehyde solutions. Neuronal tau is an important protein in promoting and stabilizing the microtubule system involved in cellular transport and neuronal morphogenesis. The significant protein tau aggregation induced by formaldehyde and the severe toxicity of the aggregated tau to neural cells may suggest that toxicity of methanol and formaldehyde ingestion is related to tau misfolding and aggregation (Chun *et al.,* 2007). Formaldehyde exposure is reported to lead to formation of DNA/protein cross links, a major mechanism of DNA damage. The DNA/protein cross links have been used as a measure of dose in drug delivery (Heck *et al.,* 1999). Formaldehyde, as a cross linking

agent, also reacts with thiol and amino groups, leading to protein polymerization (Yu *et al.*, 2006; Yu, 2001). A putative mechanism of tau aggregation in formaldehyde solution showed that formaldehyde reacts with α-/ε-amino groups and thiol groups. On the other hand, formaldehyde polymerizes itself in water (Khader *et al*., 1982).

The experiment showed that 10–15% methanol prevents tau polymerization and this suggests that tau aggregation may be due to the formaldehyde polymerization, which causes cross-linking between tau protein molecules. A tau monomer was found to first react with a formaldehyde molecule, followed by more formaldehyde molecules polymerizing and assembling onto the protein molecules. Finally, tau molecules are cross-linked by polymerized formaldehyde. The study found that such a link could not be separated even on SDS-PAGE and the mechanism of tau aggregation *in vivo* needs to be further investigated. This investigation was therefore based on the findings that tau misfolding occured in the presence of formaldehyde at low concentrations 0.01–0.1% and that methanol and ethanol were metabolized to formaldehyde and acetaldehyde respectively in human hepatocytes and some neural cells (Barceloux *et al.*, 2002; Valentine, 1990). Both formaldehyde and acetaldehyde can go through the blood-brain barrier and cause some lesions to CNS, especially human visual system (Shcherbakova, 1986). Clinically, the lethal dose of formaldehyde for human beings is reported to be about 0.08% in the circulation (Erkrat *et al.*, 1981).

The study of Chun *et al.*, (2007) has shown that formaldehyde can significantly induce tau aggregation and polymerization at concentrations even lower than 0.08% w/v, the clinical dose of toxicosis. Flitney (1966) reported the kinetics of the fixation of albumin by formaldehyde and other higher aldehydes using electron microscopy. The study found that acrolein, glutaraldehyde and crotonaldehyde were able to fix albumin far

more rapidly than the others at exposure times as short as one minute followed by formaldehyde which was able to fix albumin at a little longer time at (80 seconds). From the foregone review it could be observed that there are scanty literatures that are specific on the spectrophotometric investigation of the kinetics of the reaction of formaldehyde with proteins. Spectrophotometry however is one of the readily available analytical tool in third world countries for most studies in chemistry. This study therefore investigated the reaction of formaldehyde with plasma albumin, the most abundant proteins in blood plasma in the range of (0.01– 0.1% w/v) in aqueous and Ethanol-water media spectrophotometrically using the common various kinetic models and rate equations to add more insight to this area of global interest.

2.13 Effects of Solvent Medium on Reactions

The study of solvent effects particularly solvent-solute and solvent–solvent interactions has been of interest to many researchers (Nicotra *et al*., 2008). These interactions are found to generate new solvent properties that are absent in the pure solvent molecules which lead to phenomena such as preferential solvation that makes the nature of solute–solvents more complex (Jabbar *et al.*,2010). The influence of solvent on solute molecules has been studied intensively but the problem is far from being completely understood. However, the acceptance of a single solvent polarity scale as the most appropriate for interpreting any solvent effect has not been achieved yet. Although the exact definition of solvent polarity is still elusive, it seems reasonable to consider that this property is related to the overall solvation capability of solvent, encompassing all possible specific and nonspecific intermolecular interactions with solute ions or molecules (Reichardt, 1994).

Babak and his co-workers (2005) have also found that the medium effect on reactivity is very large and a sufficiently drastic change in medium is reported to affect not only kinetic parameters but also rate laws and mechanism and in some cases, reaction products. The rate of chemical reactions in solutions is known to be affected by two types of solvent effects: (i).Non-specific solvent effects which denote the electrostatic interaction of the solvent molecules with the ionic substances and (ii).Specific solvent effect types, which denote the chemical interaction due to the presence of electron-donating and/or electron-accepting centres in the solvent molecules. The effects of solvent on reactivity particularly alcohols as co-solvents are found to change the rate of organic, inorganic and even enzymatic reactions (Babak and Ali, 2010; Diefallah *et al.*,1978).

Several factors are found to influence these solvent effects on chemical reaction rates; these are reported to depend on: function of substrate, detailed mechanism of reaction, and experimental conditions. A solvent would not only provide a background for the reaction to occur but also stabilize the reactants and activated complex by solvating them. According to Gholami and Habibi (2000), solvent- solute interactions are much more complex in reactions involving neutral molecules and strongly solvated species. Classification of the reaction of Plasma albumin with formaldehyde according to Thanh *et al.,* (2006) can be referred to as a one-step (S_N^2) electrophile - nucleophile chemical reaction. Singh *et al*., (1968) have studied the kinetics of the oxidation of formaldehyde with neutral permanganate and found that it was base catalysed. Commonly reported reactions undertaken by formaldehyde with proteins and other biological systems include the formation of methylol adducts with proteins and the formation of stable adducts through cross-linking of labile Schiff bases with several amino acid residues.

Also the reduction of methylol alducts to methyl groups and reduction of some metal ions to their elemental forms. These reactions are found to be base catalysed and could be classified as one-step SN^2 electrophlie- nuclophile reaction (Bedino, 2003; Thanh *et al.,* 2006).

Reactions of aldehydes with alcohols are generally found to produce either hemiacetals (a functional group consisting of one – OH and one –OR group bonded to the same carbon) or acetals (a functional group consisting of two -OR groups bonded to the same carbon), depending on the conditions. Mixing the two reactants together is reported to produce a hemiacetal, while mixing the two reactants with hydrochloric acid is found to produce an acetal (Cliff, 2010).

2.14 Rate Mechanisms in Binary Mixtures

Binary mixtures are mixed solvents of water, alcohols and various organic substances that have been widely used both experimentally and theoretically to understand the type of interaction between the aqueous and non-aqueous polar molecules (Hossen *et al.,* 2012). The liquid alcohols just like water, are hydrogen bonded. Although this inter-molecular bonding has a strong influence on the properties of the liquid, it is clear that open structures are not formed as in water. Hydrogen bonding alone cannot account for the oddities in properties that are observed when alcohols are mixed with water (Jabbar *et al.,* 2010). Through hydrogen bonding and the sovent effects on the protonation constants of compounds in organic solvent – water mixtures an understanding in the biological actions of substances is achieved (Jabbar *et al.,* 2010).

Franks and lves (1966) reviewed in detail the structure of alcohol-water mixtures. From the simple view, the hydrophobic hydrocarbon chain in alcohol may be regarded as opposing the effect of the hydrophilic-OH group in its attempt to pull the molecule into solution. The hydroxyl group can form hydrogen bonds with the solvent molecules acting either as donor or as acceptor. An occurrence which becomes pronounced in certain mixed solvent systems is preferential salvation in which the solute is in contact with one solvent to a greater extent than the other (Laha *et al*. 1996). The study of solvent effect on the protonation constants of amino acids and proteins in organic solvent-water mixture for understanding their biological action has recently attracted much interest. Now, it is understood that in proteins, active site cavities of enzymes, and in different complexes of nucleotides and nucleosides the effective dielectric constant is decreased at the ligand water interface and the activity of water is decreased because of the presence of aliphatic or aromatic side chains of the ligand at the locations (Sigel, 1993). Metal ions interactions with ligands have been found to increase considerably with decreasing solvent polarity of the media. This effect is well-established in most metal ion complexes of biological ligands. Hence, knowledge of the physicochemical properties of the solvent, to understand the intermolecular interactions between solute-solvent and also solvent-solvent molecules, is required for proper bench work (Jabbar *et al.*, 2010). As for, their recognized importance, there are many experimental and theoretical works dealing with the effect of organic solvent on protonation constants of α-amino acids and peptides. The protonation constants of α- amino acids in these media are often different from those in water alone, as these media tend to be lipophilic rather than hydrophilic (Schmidt and Westheimer, 1971).

The solvation of amino acids that constitute proteins is reported to be closely connected with the stabilizing and destabilizing effects of electrolytes on protein structure. Therefore, the study of protonation and solvation processes of amino acids in various organic media is important to elucidate the connection between their chemical ability and biological activity (Carty and Hirs, 1968). The study of solvent effects particularly solvent-solute and solvent–solvent interactions has been of interest to many researchers (Banipal *et al.,* 2000.,Jabbar *et al.,* 2010). The influence of solvent on solute molecules has been studied intensively but the problem is far from being completely understood. However, the acceptance of a single solvent polarity scale as the most appropriate for interpreting any solvent effect has not been achieved yet (Jabbar *et al.,* 2010). Babak *et al.,* (2005) reported that in rate equations of organic or inorganic reactions which proceed through the formation of activated complex in binary mixtures, there is no term clearly showing the correlation between reaction rate and concentration of solvent components. In cases where none of solvent mixture components are reactant, a study involving a series of experiments in which the concentrations of mixture components were considered, their concentration was found to be no more constant. Their study also found that when one of solvent components acts as a reactant, the rate equations consider no distinction for it as a solvent reactant.These workers introduced a new way for study of one - step chemical reactions in binary mixtures by which the role of solvent system in raising or lowering the reaction rate is inserted in the reaction rate equation. This model was called "study of effect of solvent mixture on the one – step reaction rates using the transition state theory and cage effect" (SESMORTAC Model).

This model by Babak *et al.,* (2005) is based on the following assumptions:

1. One – step reaction in binary mixture solution proceeds simultaneously by two parallel reactions. The first mechanism is similar to the reaction mechanism in pure solvent, while in the second mechanism the added solvent is involved in the formation of activated complex as well. The total reaction rate is found to be the sum of these two reactions.
2. For calculating the concentration of activated complex formed in the second mechanism (ACSM), it is assumed that with the beginning of the interaction between the interacting species, say A and B we would face a chemical species (A-B), called pseudo-collision complex. These molecules are viewed as being surrounded by a cage formed by solvent molecules. Cage effect is reported to keep the molecules of A and B close together for relatively long time, during which they collide repeatedly with each other and with the cage walls of solvent molecules (Benesi, 1982). The detailed reaction steps are as represented in equations (2.3-2.6).

   The reaction in the second mechanism is reported to occur in two steps as follows: The first step is

$$[(A+B).nS_1]cage \rightarrow [(A..B).nS_1]^{\neq}cage \quad ---------- 2.3$$

Pseudo - collision complex

The primary interaction would insignificantly raise the potential energy. This assumption is considered in accounting for the total solvent effects from the beginning of interaction between interacting species to formation of ACSM.

The second step is:

$$[(\mathrm{A..B}).\mathrm{nS_1}]^{\neq}\mathrm{cage} + \mathrm{n\ molecules\ of\ S_2\ solvent} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$[(\mathrm{A..B}).\mathrm{nS_2}]^{\neq}\ \mathrm{cage} + \ \mathrm{n\ molecules\ of\ S_1\ solvent} \xrightarrow{k_2} \mathrm{product} \quad --- 2.4$$

ACSM

According to these authors in the process of formation of ACSM, from pseudo - collision complex in binary mixture solution of S_1 and S_2 solvents , the *n* molecules of S_1 (in the cage or solvation shell of pseudo collision complex) would be replaced by *n* molecules of S_2 (from bulk solution). Using the steady state approximation for calculation of ACSM concentration, the reaction rate equation would be given upon the concentration of binary mixture components. Thus the overall reaction is the sum of steps (1) equation (2.3) and (2).(equation, 2.4).

$$[(A+B).nS_1]cage + n\ molecules\ of\ S_2\ solvent \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$[(A..B).nS_2]^{\neq}\ cage + n\ \ molecules\ of\ S_1\ \ solvent \ \overset{k_2}{\rightarrow}\ product \quad ------ \quad 2.5$$

ACSM

and the rate equation in binary mixture is

$$v = k_{obs}[A][B] \quad --------- \quad 2.6$$

where k_{obs} is the observed second order rate constant in solvent mixture.

There are some reported limitations in determining the acidity constants of the molecules such as low solubility in aqueous solutions and the low values of acidity constants. Acid dissociation constant (p*Ka* value) is an important parameter in the estimatimation of the extent of ionization of drug molecules at different pH values, which is of fundamental importance in the consideration of their interaction with biological membranes. Therefore, to enhance the acidity constants on one hand and to increase the solubility on the other, researchers are forced to choose mixed solvents (Hemmateenejad *et al.,* 2008). In addition, the p*K*a of the analytes in the mixed solvent is an important parameter for the optimization of separation techniques such as capillary electrophoresis and liquid chromatography of ionisable compounds (Sanli *et al.,* 2010). An understanding of the factors that determine the acidity or basicity of a solute solved in solvent mixtures, can also be decisive to foresee how the aqueous p*K*a is modified

when the organic modifier is added to the aqueous part of the eluent for separation methods and in order to establish relationships allowing to relate an aqueous p*K*a with the p*K*a in the organic modifier-water binary mixtures.

Moreover, the study of acid-base behavior of analytes in binary organic-water solvent systems could be key in predicting influence of pH on retention and selectivity in liquid chromatography (Jimenez-Lozanoet *et al.*, 2002). Kaufman *et al.*, (1975) reported that organic solvents such as ethanol, acetone, acetonitrile (MeCN) and their mixtures with water, are widely used in separation techniques due to the excellent characteristics of the pure solvent. Acetonitrile (MeCN) for example is a very weak base and a very weak acid and therefore it is a good differentiating solvent for both acids and bases. Furthermore, it has low viscosity and ideal good ultraviolet/visible transparency. It has a relatively high dielectric constant ($\varepsilon = 36$) and a small autoprotolysis constant (p*K*s = 33.6).

2.15 The Efect of Dielectric Constant on the Reaction Rate

Seyhan, (2008) reported that the solvent used in substitution reactions was found to inherently determine the nucleophilicity of the nucleophile and this fact has become increasingly more apparent as more reactions are performed in the gas phase. As such, solvent conditions significantly affect the performance of a reaction with certain solvent conditions favouring one reaction mechanism over another. For S_N1 reactions the solvent's ability to stabilize the intermediate carbocation is of direct importance to its viability as a suitable solvent. The ability of polar solvents to increase the rate of S_N1 reactions is a result of the polar solvent's solvating the reactant intermediate species, i.e., the carbocation, thereby decreasing the intermediate energy relative to the starting material (Seyhan, 2008).

Huges and Ingold (1953) however found that the case for S_N^2 reactions is quite different, as the lack of solvation on the nucleophile increases the rate of an S_N^2 reaction. In either case (S_N^1 or S_N^2), the ability to either stabilize the transition state (S_N^1) or destabilize the reactant starting material (S_N^2) acts to decrease the $\Delta G^{\ddagger}$ of activation and thereby increase the rate of the reaction. This relationship is according to the equation (2.7)

$$\Delta G^{\ddagger} = - RT \ln K \qquad \text{(Gibb's free energy)} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2.7$$

The rate equation for S_N^2 reactions are bimolecular being first order in nucleophile and first order in reagent. The determining factor when both S_N^2 and S_N^1 reaction mechanisms are viable is the strength of the nucleophile. Nuclephilicity and basicity are linked and the more nucleophilic a molecule becomes the greater said nucleophile's basicity. This increase in bacisity causes problems for S_N^2 reaction mechanisms when the solvent of choice is protic. Protic solvents react with strong nucleophiles with good basic character in an acid/base fashion, thus decreasing or removing the nucleophilic nature of the nucleophile. Tables 2.2 - 2.4 show the effect of solvent polarity on the relative reaction rates of the S_N^2 reaction of n-butyl bromide with azide, N_3^-. Note the gross increase in reaction rates when changing from a protic solvent to an aprotic solvent. This difference arises from acid/base reactions between protic solvents (not aprotic solvents) and strong nucleophiles. It is important to note that solvent effects as well as steric effects both affect the relative reaction rates; however, for demonstration of principle for solvent polarity on S_N^2 reaction rates, steric effects may be neglected. There are two major factors affecting the reactivity of a specific amino acid side chain toward a reagent that under ideal conditions reacts with it.These have effects on the pK

of the reacting group (which affects its nucleophilicity), and steric effects (Seyhan, 2008).

2.16 pK Values of Some Amino Acids Reacting Groups

The pK of a chemical species is its acidity constant (p*K*a). A number of factors influence the pK of an ionizable amino acid side chain in a protein, these include the electrostatic field of the protein (especially the local charge distribution around the particular side chain), the solvation of the group and the hydrogen bonds, if any, that the group may be involved in. An examination of the pK values commonly found in protein side chains have been reported to be in a range from approximately 3.75 to > 12 Berg *et al.,* 2002**)** as presented in Appendix C

Table 2.2: The relative solvolysis rates of *tert*-butyl chloride with acetic acid, methane and water

Solvent	**Dielectric constant ε**	**Relative Rate**
CH_3CO_2H	6	1
CH_3OH	33	4
H_2O	78	150000

Table 2.3: The effect of solvent polarity on the relative reaction rates of the S_N^2 reaction of n-butyl bromide with azide, n_3^-

Solvent	Dielectric constant ε	Relative rate	Type
CH_3OH	33	1	Protic
H_2O	78	7	Protic
DMSO	49	1,300	Aprotic
DMF	37	2800	Aprotic
CH_3	38	5000	Aprotic

Table 2.4: Comparison between calculated and experimental dielectric constants (ε)

%Solvent		Calculated (ε)	Experimental (ε)
Water	**Ethanol**		
Pure	**-**	**80.0**	**79.04**
-	**Pure**	**24.6**	**23.6**
83.3	**16.6**	**70.9**	**68.8**
50	**50**	**52.4**	**51.2**
20	**80**	**35.7**	**35.4**

Data from Khalil *et al*, 2012

2.17 Determination of a Ligand- Ligand or Ligand to Metal Ratio

UV-Vis spectroscopy is very useful in determining the ratio between a ligand – ligand or ligand and a metal as given in the relation below:

$$M^{n+} + m\,L = (ML_m)^{n+} \quad \text{---------- 2.8}$$

Usually, complexation of ligands with metals result in different spectroscopic characteristics for both. The most pronounced situation is the formation of a coloured complex.

2.17. 1 The method of continuous variation (Job's method)

In this method, the mole fraction of either the metal or the ligand is plotted against absorbance.as given in equation (2.9).

$$\text{Mole fraction} = \frac{\text{mol } M^{n+}}{\text{mol } M^{n+} + \text{ mol L}} \quad \text{– – – – – 2.9}$$

Tangents are drawn on both side of the maximum obtained and a perpendicular line is drawn to the axis representing the mole fraction. This gives the mole fraction of the metal. If the value is 0.5 then the stoichiometric ratio of the complex is a 1:1 and if it is 0.33 then it is a 1:2 stoichiometric ratio etc.The method of continuous variation is found to be excellent for complexes that are 1:1 but if the ratio is more than 1:2 there will be some considerable uncertainty and the mole ratio method is preferred (Huang, 1982).

2.17.2 Mole ratio method

The concentration of the ligand, or metal ion is usually kept constant and a variable amount of the complexing agent is added. The mole ratio of the metal ion to the ligand is plotted versus absorbance. Tangents are drawn and a perpendicular line is drawn to the mole ratio axis showing the exact ratio

(Chriswell and Schilit, 1975). Models of continuous variation and mole ratio methods are as presented in Appendices C and D.

2.18 Formaldehyde-Plasma Albumin Adduct as a Glycoprotein (Lectin)

Lectins are glycoproteins of plants and animals origin that recognize and bind to specific oligosaccharides. Common examples of lectins include: Concanavalin A and wheat germ agglutinin which are plant lectins that have been found as useful research tools, the C-type lectin-like domain which is a Ca^{2+} binding carbohydrate recognition domain in many animal lectins. Recognition/binding CHO moieties of glycol proteins, glycolipids and proteo glycans by animal lectins. These are reported to be factors in: cell-cell recognition, adhesion of cells to the extra cellular matrix, interaction of cells with chemokines and growth factors, recognition of disease causing micro organisms, initiation and control of inflammation. Other examples of animal lectins include Mannan-binding lectin (MBL) which is a glycoprotein found in blood plasma. It binds cell surface carbohydrates of disease-causing micro-organisms and promotes phagocytosis of these organisms as part of the immune response (Diawan, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Reagents

Paraformaldehyde (90%w/v)

Formaldehyde (37- 40% w/v,1.085 g/cm^3)

Absolute Ethanol (90-99 %v/v, 0.98g/cm^3), BDH Poole, England.

Acetoacetalinide ($C_{10}H_{11}NO_2$, 99.5%), Sigma-Aldrich , Germany.

Acetylacetone ($C_5H_8O_2$, 99.5% , 0.97g/cm^3) Sigma-Aldrich , Germany.

Ammonium acetate ($C_2H_6NO_2$), BDH Poole, England

Ammonium citrate (97%), Analytical reagent M&B lab chemicals Dagenham, England.

Glacial acetic acid: (99.85%, 1.055 g/cm^3) Analytical reagent M&B lab chemicals, England

Potassium iodide (KI), 99.8% , Sigma-Aldrich, Germany.

Acrylamide , BDH

Diethyl ether BDH Poole, England.

Chloroform (Trichloromethane) 60 - 62%: Analytical reagent M&B lab chemicals

Sodium citrate M&B

Potassium iodate, Sigma-Aldrich, Germany.

Phosphorus pentoxide, BDH Lab reagent, Poole, England.

Sodium thiosulphate, Sigma, Germany.

Sodium Hydroxide

Sodium Chloride

Sodium azide, Koch - light Laboratories Ltd.

Hydrochloric acid

Plasma abumin, isolated and purified form.

Sodium Sulphite AnalaR,

3.2 Methods

3. 2.1 Sampling

The human blood samples analysed in this study were obtained from the Federal Medical Centre, Makurdi after obtaining an ethical committee approval. The human urine and milk samples which were early morning samples were donated freely by persons who were committed to the progress of this study at the Benue Stae University Clinic, Makurdi. The samples were taken in K_2EDTA sample bottles previously sterilized for such purpose and subsequently taken to the laboratory for isolation and purification of the plasma albumin used in this study. Foods, cosmetics and non-food materials products were also collected and analysed in this study**.** The food products were procured randomly from Wurukum and Wadata Markets in Makurdi- Benue State. They included, Raw Human milk, Liquid and powdered Peak milk, Nunu milk, Raw Cow milk, Fresh fish (local), Frozen fish (mackerel), Smoked fish (mackerel), Fried fish (mackerel and dried fish (local). The cosmetics and non-food samples analysed in this study were procured from different parts of Gboko market and for each of the samples, three different brands were collected for investigation. The sample materials collected and analysed are as listed in Tables 3.1 and 3.2 respectively.

Table 3.1: The body fluids, fish and milk samples

SNo.	Samples
1.	Human blood (composites)
2	Human Urine (composites)

3	Human milk (composites)
4	Raw Cow Milk
5	Peak Milk (P)
6	Frozen fish (Mackerel)
7	Nunu milk (P)
8	Dry fish (local)
9	Fried fish (Mackerel
10	Smoked fish (mackerel
11	Fresh fish (local)

P= Powdered, L = liquid

Table 3.2: The cosmetics and the non-food materials

Cosmetics	Non - food materials
Lipstick 1	Growers 1

Lispstick 2	Growers 2
Lispstick 3	Layers
Petals 1	Napkin1
Petals 2	Napkin2
Sheen 3	Napkin3
Toilet Tissue 1	Toilet Tissue 3
Toilet Tissue 2	

3.2.2 Extraction of formaldehyde from human urine, milk, foods, cosmetics and non – food materials

Water based materials were dissolved with water, while solvent type substances were dissolved with ethyl acetate and then water added and the free formaldehyde dissolved in water was distilled under acid condition. .Formaldehyde was extracted from the

food substances by steam distillation and extraction with ether. Before extraction the foodstuffs were homogenized (AOAC, 1984).

3.3 Isolation and Fractionation of Plasma Albumin from Human Whole Blood using Ethanol as Organic Solvent.

This study employed ethanol and 0.14% HCl acid to undertake the isolation and fractionation of whole human blood to obtain plasma albumin with the hope of obtaining high purity and as a way of exposing the efficacy of Ethanol as another of the organic solvents that can be used to isolate plasma albumin using the various listed methods and produres :

3.3.1 Removal of haemoglobin from the blood samples

The human blood samples were pre-treated to remove haemoglobin the major contaminant in human plasma albumin, fibrinogen, and globulin using ethanol and 4% HCl based on the methods of Tanaka *et al.,* (2001) and Michael , 1968,) as follows: 600cm^3 of the whole blood samples of pH 4.0 were treated in three batches. Each of the batches was taken in K_2EDTA sample bottles and centrifuged for 30 minutes at 2500 r/min. Then the plasma fraction was carefully pipetted out using a teat pipette into a pre weighed beaker 215 cm^3 and was finally weighed to obtain a 183.72g plasma sample. Then the pH of the plasma sample was adjusted to 7.2 with acetate buffer, (4 mol dm^{-3}) nsodium acetate and 10 mol dm^{-3} acetic acid of pH 4.0). Next, 20cm^3 of a mixture of 19% v/v cold ethanol as well as containing 0.6 % v/v tetrachloromethane was slowly added under homogenization. This mixture was kept at 4.0 ^{0}C in a refridgerator for 30 minutes and further homogenized for 20 minutes in order to achieve complete haemoglobin precipitation.

3.3.2 Removal of α- and ß- globulin

After the haemoglobin precipitation, the plasma albumin aliquot was left overnight in a refrigerator at 0^0C for ɣ-globulin precipitation. The precipitate consisting of haemoglobin and ɣ-globulin were then removed by centrifugation at 2,500 r/min for 10 minutes and was discarded to obtain a supernatant I. The pH of the supernatant I aliqout was adjusted to 6.0 with acetate buffer solution of pH 4.0 under homogenization for 30 minutes and the precipitate left to stand for 3-6 hr during which the α- and ß-globulin precipitates were formed. These were removed by centrifugation as described above and discarded to obtain an aliquot hereafter referred to as supernatant

3.3.3 Precipitation of pure albumin protein

The supernatant II was then mixed at room temperature with $10cm^3$ of 90% ethanol containing 0.05 mol dm^{-3} hydrochloric acid while stirring. After 30 minutes of vigorous stirring the mixture was centrifuged, the liquors were removed, and the precipitated globulins washed in the centrifuge with a volume of 90% ethanol equal to half the original volume of serum or plasma. The liquor and washings were combined to obtain a clear solution. The clear solutions thus obtained were neutralized with $10cm^3$ of 0.5 mol dm^{-3} NaOH to an apparent end point of pH 6.0, whereby a white flocculent precipitate of albumin was formed. The precipitated albumin was allowed to settle and was freed from the liquor by decantation and centrifuging. It was successively, washed twice each while still in the centrifuge with ethanol and diethyl ether respectively.The precipitate was dried in air and finally in vacuo over P_2O_5 at room temperature and weighed. The albumin was thus obtained in the form of a free-flowing, non-hygroscopic powder.

3. 3. 4 Characterization of isolated plasma albumin

The isolated and purified plasma albumin from whole blood was characterized using Protein analysis, Solubility test**,** Denaturation tests and Optical rotation measurement which are the simple and available methods.

3. 3. 5 Protein analysis by Kjeldahl method

The protein contents of the albumin preparations was determined using the Kjeldahl method and the protein was calculated from the nitrogen figures by using the factor 6.25 as follows: The samples were digested with 20cm^3 of concentrated H_2SO_4, 150cm^3 of molar NaOH (40%), 10g of K_2SO_4 and 0.5g of $CuSO_4$. Five (5cm^3) of the digest was placed into a micro-kjeldahl distillation apparatus and 150cm^3 of the (40%) NaOH was added to make the solution strongly alkaline. The Ammonia was distilled into 5 cm^3 of boric acid indicator in a titrating flask until more than 45 cm^3 of the distillate was collected. Titration was done with 0.01 mol dm^{-3} HCl to obtain a light green end point (AOAC, 1980)

3.3.6 Solubility test

The solubility test on the albumin samples was carried out based on the method of Ehrenpreis *et al., (*1957) as follows: 0.005g of the albumin samples were mixed with 100cm^3 water to give a 0.005% solution of the albumin, then 0.1 moldm^{-3} NaOH was added drop by drop until the pH was 7.4. The rate and completeness of dissolution were observed. The same procedure was carried out using commercially processed egg and bovine albumin samples and the rate of dissolution observed (Ehrenpreis *et al.,* 1957).

3. 3. 7 Denaturation tests

The appearance of turbidity or precipitate when 0.1mol dm^{-3} NaOH is added to a protein sample is a measure of its native form. When precipitation or turbidity occurred it is usually taken as an indication of some degree of denaturation of the protein. Invariably, any such turbidity or precipitate is reported to re-dissolve at pH 7. This test, in which the solubility of albumin at different pH values is examined, is similar to one of the methods described as a criterion for the native state or degree of denaturation of albumin. The denaturation test on the plasma Albumin samples was carried out as follows: 0.01g each of the isolated plasma albumin sample along with commercially processed Egg and Bovine albumin samples were taken in a beaker and $5cm^3$ of 0.1 mol dm^{-3} NaOH were added to each beaker at varying pH of 5.0, 6.0, 7.0 and 8.0 and was allowed to stand for ten minutes. After the time interval each of the samples was examined for appearance of precipitate or turbidity, (Ehrenpreis *et al.,* 1957).

3. 3. 8 Optical rotation measurement of the albumin samples

In this study, (0.005%, w/v) concentration of the albumin samples, were adjusted accurately to the pH 7.4 by the addition of 0.1 mol dm^{-3} NaOH, and examined for their optical rotation,$[\alpha]_D^t$ based on the method of WHAR 3.10 (1977) as follows:

$20cm^3$ of distilled water was measured in the observation tube of the polarimiter and the blank reading observed and recorded that is when maximum darkness occurred and was recorded as angle of rotation theta , θ_1. The distilled water in the observation tube was replaced with the albumin sample solutions (0.005g) of Bovine Albumin, Egg York Albumin, and the two isolated Plasma Albumin samples respectively and again the

angle of rotation (θ_2) of each sample was measured and recorded. The difference (θ_{2-} $\theta_{1)}$ was recorded as the angle of rotation. Triplicate readings for each of the four samples were made and the angle of rotation obtained from the relation (θ_{2-} θ_1).

3.4 Preparation and Characterization of 1,4-Dihydropyridine Adduct from Formaldehyde, Acetoacetanilide and Plasma Albumin

The 3,5-diaphenyl,1,4-dihydropyridine adduct was prepared from a mixture of acetoacetanilide, formaldehyde and plasma albumin and was characterized using melting point, IR, Uv spectra characteristics as well as the microbial activity based on the method described by Hantzsch method as follows: A mixture of 20cm^3 of acetoacetanilide (0.02 mol dm^{-3}) , 20 cm^3 of (0.0001 mol dm^{-3}) formaldehyde and 10cm^3 of (0.0001 mol dm^{-3}) plasma albumin were taken in a reflux flask and refluxed at a temperature of 25.0 ^{0}C for one hour on a water bath. The contents were transferred in a beaker and left to stand overnight after which it was filtered to obtain the yellow dihydrolutidine derivative.The adduct was recrystallized from water and dried over potassium permanganate in a desiccator. The prepared adduct was analysed for its melting point, maximum absorption using uv/visible spectrophotometer and its functional groups using Fourier transform infrared spectroscopy as well as its antimicrobial susceptibility test (Li *et al*.,2007). The reaction scheme of the proposed adduct is as presented in (Figure 3.1).

3. 4.1 Fourier transform infrared spectroscopy

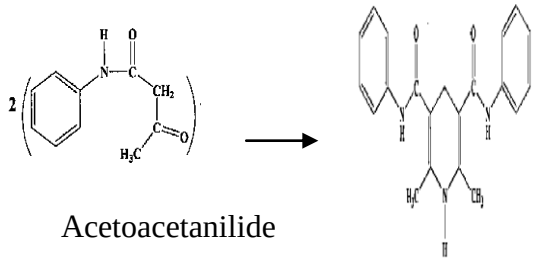
The Fourier Transform Infrared spectroscopy (FTIR) analysis of the adduct was carried out on a Nicolet IR spectrometer (IR 100) in the range of 400.75 – 400 cm^{-1} at 95.586 runs per minute in a KBr pellet mulling agent. The data chart was then plotted

on an IBM computer using win FIRST plot composer software. The percentage transmittance bands and intensities were then read from the chart.

3. 4.2 Melting point analysis

A weighed quantity of the sample (0.2g) was taken in a capillary tube that was previously sealed at one end and the capillary tube inserted into a 9100 BI Barmstead electro thermal machine and was turned on. The sample tube was observed through the magnifying lens over the automated temperature ranges on the machine.

The temperatures at which the sample in the capillary tube began to melt and that which it finally melted was observed and recorded. Triplicate readings were taken and the results recorded.



$HOC(CO_2H)(CH_2CO_2NH_4)_2$ + HCHO +

Ammonium citrate Formaldehyde Acetoacetanilide

+ $HOC(COOH)(CH_2COOH)_2$+ H_2O ----------3.1

3,5-diphenylamine-1,4dihydro-2,6 dimethylpyridine dicarboxylate + 3-hydroxylpent-2,3,4-tricarboxylicacid + water

Figure 3.1 Structure of the proposed reaction scheme of acetoacetanilide and formaldehyde in the presence of ammonia aerived from ammonium citrate to form diphenylamine-1,4dihydro-2,6 dimethylpyridine dicarboxylate (Sigma Aldrich , 1984).

3.4.3 Antimicrobial screening test

Antimicrobial screening of the adduct was determined using agar well diffusion method (Nair *et al*., 2006). The investigated organisms were *Bacillus subtilis, Proteum mirabilis, Canidad albicans, Escherichia coli* and *Staphylococcus aurus*. These organisms were cultured into prepared normal saline and were incubated for 30 minutes at 37 ^{0}C. The population of each of the organisms was increased to form a turbidity that marched with 0.5 McFarlan‘s standard by visual comparison at which it is assumed that the number of cell is 1.5x10 cfu/mL. The cell suspensions were seeded into the plate of nutrient agar. Wells were bored into the plate of the seeded micr-organisms using sterile cork borer of 6mm in diameter.The adduct(mg/mL) was constituted in distilled water. This concentration was introduced into each of the wells and was allowed to

stand for 30 minutes at 40^0C for proper diffusion of the adduct. Alm the plates were fully incubated at 37^0C for 24 hr and the zones of inhibition diameter in millimeters were measured and recorded (Nair *et al*., 2006).

3. 5 Preparation of Samples and Reagents

All the reagents used in this study were of analytical grade hence no further purifications were carried out except the paraformaldehyde stock reagent which was procured in solid form that was further distilled for a pure formaldehyde. The test samples were properly handled right from the collection point where they were kept in sterilized K_2EDTA sample bottles and analysed immediately after sampling in the laboratory.

3.5.1 Distillation of paraformaldehy (90% w/w, 1.085, 32.52 mol dm^{-3}) to obtain pure formaldehyde solution

The stock aqueous formaldehyde was prepared by distilling 2.0 g dm^{-3} of the 32.52 M Paraformaldehyde which was dissolved with deionised water in a conical flask. The distillation was carried over a fractional distillation unit until about 200 cm^3 volume of the formaldehyde was recovered. The pure formaldehyde aliquots collected were further standardized using known standard methods to acertain the correct concentration of the aqueous formaldehyde solution.

3.5.2 Standardization of the purified formaldehyde solution by iodometric method

The recovered formaldehyde (10cm^3) were taken into a 250 cm^3 flask and a portion, 10.0 cm^3 of 0.1 mol/L iodine solution was added. Then 1.0 cm^3 of a 5.5 mol dm^{-3}

sodium hydroxide solution was added drop by drop until the colour fades to pale yellow. Next 2.0 cm^3 of 6 mol dm^{-3} hydrochloric acid solution were added and the mixture left to stand for ten minutes in the dark. The standardization process was continued by further addition of 100cm^3 of freshly - boiled and cooled water and the sample then titrated to a pale yellow colour with standardized sodium thiosulfate solution. Finally a solution of freshly prepared 1 g/ cm^3 starch indicator (1.0 cm^3) was added and the titration continued to the end point indicated by the of disappearance of the blue colour. The blank was also tested at the same time (Day and Underwood, 1991).

The concentration of formaldehyde standard stock solution was calculated as follows:

$$C_{formaldehyde} = \frac{(V_1 - V_2)C\ x\ 30.03}{5.0} - - - - - 3.2$$

Where

$C_{formaldehyde}$ = Concentration of formaldehyde in g dm^{-3}

V_1= Consumed sodium thiosulfate solution by the blank, in centimetres (cm^3);

V_2 = Consumed sodium thiosulphate solution for formaldehyde standardization, centimetres (cm^3).

C = Concentration of the sodium thiosulfate solution, in moles dm^{-3}

30. 03 = Molar mass of formaldehyde

5.0 = Sampling size of the formaldehyde standard stock solution, in cubic centimeters.

3.5.3 Preparation of formaldehyde working solutions

The formaldehyde concentration in this study was selected in range of (0.02 – 0.1% w/v) for investigation from below and above the reported toxic limit of 0.027 $moldm^{-3}$ of aqueous formaldehyde in the blood. The 6.7 $x10^{-2}$ mol dm^{-3} stock aqueous formaldehyde was further diluted by serial dilution method to prepare 2.0 $x10^{-2}$, 2.7

$x10^{-2}$, and 3.6 $x10^{-2}$ mol dm^{-3} (0.6 -1.0 g dm^{-3}) working aqueous and 20% w/v ethanol-water formaldehyde working standards by diluting calculated volumes of the stock aqueous formaldehyde solution into $10cm^3$ volumetric flasks and the final volume made up to the mark with de ionized water. The dilute standard solutions were freshly prepared before each of the experiments.

3.5.4 Preparation of plasma albumin working solutions

The stock solutions of the plasma albumin used in the study were prepared by dissolving $50gdm^{-3}$ = 0.75 $x10^{-3}$ mol dm^{-3} with distilled water. The aqueous stock solution was prepared by diluting 50g of the plasma albumin in a volumetric flask followed by addition of $10cm^3$ of (0.1mol dm^{-3} HCl) and the mixture was further diluted to mark in a $1000cm^3$ volumetric flask with distilled water. Other lower albumin working solution of the plasma albumin (40 gdm^{-3}) was also prepared by the dilution of 1.6 cm^3 of the 0.75 $x10^{-2}$ mol dm^{-3} stock solution in a $10cm^3$ volumetric flask to obtain the 0.6 $x10^{-3}$ mol dm^{-3} working albumin solution.

3.5.5 Preparation of Percentage Ethanol-Water Mixtures

$$\%\frac{w}{v}\text{Ethanol} = \frac{\text{Volume of ethanol x 100}}{\text{Volume of sample}} \quad ---------- 3.3$$

$$\text{Volume of ethanol} = \frac{\%\ \text{Volume of ethanol xvolume of sample}}{100} \quad --3.4$$

Using equations (3.3 and 3.4) varying percentage ethanol-water mixtures were prepared by measuring known volumes of a 17.04 mol dm^{-3} ethanol stock solution (mwt. 46.07, s.g 0.7930) into series of 50 cm^3 volumetric flasks and the contents were made to mark with distilled water. Using this procedure 0.5 cm^3, $2.5cm^3$, 5.1 cm^3, 7.6 cm^3 , 10.1 cm^3 and 12.6 cm^3 were measured into 50 cm^3 volumetric flasks and the contents

were made to the mark with distilled water to obtain 1%, 5% ,10%. 15%, 20% and 25% ethanol-water mixtures respectively.

3.5.6 Preparation of percentage ethanol – water mixtures of varying dielectric constants

According to the method of Deosarkar and Narwade, (2009), the dielectric constant of various percentage ethanol-water mixtures (1-25%v/v) were prepared by addition of some quantity of stock ethanol (17.04 mol dm^{-3}) with distilled water and the dielectric constants were calculated from the relation.

$$D_{medium} = \frac{\%ethanol \times D\ ethanol + \%water \times Dwater}{100} \quad \text{----------3.5}$$

3.5.7 Preparation of hemiacetals from (1-25%w/v) ethanol - water mixtures and formaldehyde concentrations

The hemiacetals were prepared readily by method of Tong, (2003) by dissolving the various formaldehyde concentrations in the various (1-25) % ethanol-water mixtures in 1:1 ratio and the stock solution left to stand for two hours in some beakers. Fresh preparations were made for each runs.

3. 5. 8 Preparation of acetylacetone - ammonium citrate buffer solution (2.0 w/v)

The Acetylacetone –Ammonium citrate buffer was prepared based on the method of Nafisur *et al.*, (2007)) as follows: 4.86 g of ammonium citrate was weighed and dissolved with small quantity of water, 3 cm^3 of glacial acetic acid and 0.25 cm^3 of acetylacetone was added, properly mixedl and made up to 100 cm^3 with water and the pH adjusted to 7.5 with drop wise addition of 0.1 mol dm^{-3} NaOH.

3.5. 9 Preparation of ammonium citrate (0.2 mol dm^{-3})

This stock solution was prepared by dissolving 12.16g of ammonium citrate in a beake with distilled water followed by addition of 3 cm^3 of acetic acid and diluting it to the mark into a 250 cm^3 volumetric flask (pH 7.5).

3.5.10 Preparation of aqueous hydrochloric acid solutions HCl (0.05 and 6.0 mol dm^{-3})

These were prepared by diluting 0.95 cm^3 and 128.88 cm^3 of concentrated hydrochloric acid (s.g 1.18, % purity36) with distilled water in two separate 250cm^3 volumetric flasks and the contents were made up to mark.

3 .5.11 Sodium hydroxide solutions NaOH (0.1 and 7.5 mol dm^{-3})

The NaOH solutions were prepared by weighing 1.0 and 30g of sodium hydroxide pellets separately and were dissolved in two 100 cm^3 beakers with distilled water. The contents of each beaker was transferred quantitatively two into 250cm^3 flasks and each diluted to mark to the produce 0.1 and 7.5 mol dm^{-3} sodium hydroxide solutions respectively.

3.5.12 Preparation of sodium chloride solutions NaCl (1.0 and 3.9 mol dm^{-3})

These were prepared by dissolving 14.65g and 57.04g of anhydrous sodium chloride into two separate 100cm^3 beakers with small quantity of distilled water and each beaker was quantitatively transferred into two 250 cm^3 volumetric flask and diluted to mark to obtain a 1.0 and 3.9 mol dm^{-3} solutions respectively.

3. 5.13 Preparation of potassium iodate KIO_3 (0.05 mol dm^{-3}).

KIO_3 (2.68 g) was weighed into a 100cm^3 beaker by use of a funnel. The funnel was properly rinsed with some distilled water and the KIO_3 solution quantitatively transferred into a 250 cm^3 volumetric flask and diluted to the mark and mixed thoroughly.

3 .5.14 Sodium thiosulphate ($Na_2S_2SO_3$, 0.1 mol dm^{-3})

Some 100cm^3 of distilled water were boiled for 10 minutes and was allowed to cool to room temperature. Then, 25g of $Na_2S_2SO3.\ 5H_2O$ and 0.1 g of Na_2CO_3 were added and stirred until the solids were dissolved and the volume was made up to the mark in a 250cm^3 volumetric flask. The solution was then transferred to a clean glass or plastic bottle and stored in a dark place.

3.5.15 Starch indicator solution (0.5%)

Starch indicator solution (0.5%) was prepared by method of College of Science, (2011) as follows: 0.25 g of soluble starch was weighed and added into a 50cm^3 of near boiling water in a 100 mL conical flask. The content of the flask was stirred to dissolve and cooled before use.

3.5.16 Iodine solution (I_2, 0.005 mol dm^{-3})

The Iodine solution was prepared by the method of College of Science, (2011) by weighing 2.0 g of potassium iodide and 1.3 g of iodine into a 100 cm^3 beaker. Then 20 cm^3 of distilled water was added and swirled for a few minutes until the iodine was dissolved. The iodine solution was transfered into a 1000cm^3 volumetric flask to the

mark, making sure to rinse all traces of solution into the volumetric flask using distilled water.

3.5.17 Acetic acid 10 mol dm^{-3} (2.07, 99.9 %)

The acetic acid stock solution was prepared by measuring 290.17 cm^3 of analytical grade glacial acetic acid into a 1000 cm^3 volumetric flask and was diluted to the mark with distilled water.

3.6 Preparation of Formaldehyde Calibration Curve and Determination Formaldehyde Levels in the Samples using Acetylacetone Reagent

The calibration curve for formaldehyde was prepared from five points over standard solutions of formaldehyde in the range of (0.67×10^{-2} - 0.33×10^{-1} mol dm^{-3} (0.02- 1.0g dm^{-3}) using acetylacetone reagent based on the method of Nafisur *et al.,* (2007) with some modifications whereby ammonium citrate was used in place of the reported ammonium acetate as follows: A portion (100 cm^3) each of aqueous solution of formaldehyde (0.67×10^{-2} - 0.33×10^{-1} mol dm^{-3}) was pipetted into a 100cm^3 volumetric flsk followed by the addition of 20cm^3 of the acetylacetone – ammonium citrate buffer and the volumes made to the mark with distilled water. The content of the mixture was kept in a constant temperature thermostatic water bath at 40 ^{0}C for five minutes. The absorbance of the yellow coloured product was then measured at 415nm against distilled water reagent blank. Using the the same procedure as above the absorbances of the samples namely human urine, milk and food samples as well as the cosmetics and non-food samples were measured and the concentration of the

formaldehyde in the samples in mol dm^{-3} and g/kg respectively were determined from the calibration graph (Li *et al.,* 2007). The calibration curve is as presented in Appendix D.

3.6.1 Preparation of formaldehyde calibration curve and determination of formaldehyde levels in the samples using acetoacetanilide reagent

The calibration curve for formaldehyde was prepared from five points over standard solutions of formaldehyde in the range of (0.67×10^{-2} - 0.33×10^{-1}mol dm^{-3} (0.02 - 1.0g dm^{-3}) using Acetoacetanilide reagent based on the method of (Li *et al.,* 2007) as in the acetylacetone reagent as follows:

A portion (100 cm^3) each of aqueous solution of formaldehyde (0.67×10^{-2} - 0.33×10^{-1} mol dm^{-3}) was pipetted into a 100cm^3 volumetric flsk followed by the addition of 20cm^3 of the Acetoacetanilide – ammonium citrate buffer and the volumes made to the mark with distilled water. The mixtures were kept in a constant temperature thermostatic water bath at room temperature and left to stand for five minutes. The absorbance of the yellow coloured product formed for each flask was then measured at 368nm against the reagent blank. Using the same procedure the concentration of the free formaldehyde in all the samples namely ; human milk, urine and food samples as well as the cosmetics and non-food samples were measured and the concentration of the free formaldehyde in the samples were calculated in moldm^{-3} and g/kg respectively. .The calibration curve is as presented in Appendix D.

3. 7 Kinetics Studies

The rates of reactions were monitored on a Uv-visible Spectrophotometer (Jenway 6305 uv/vis spectrophotometer model). The rate of reaction of formaldehyde in water and ethanol-water mixtures with plasma albumin was studied by monitoring the

decrease in absorbance of the formaldehyde – plasma albumin adduct formed at 235nm. All kinetic measurements were carried out under pseudo-first order conditions with formaldehyde concentrations in at least 500, 100 and 50 fold excess over plasma albumin concentrations in the water solution and ethanol-water mixtures.The $[H^+]$, ionic strength and temperature of each reaction system was kept constant. The Pseudo-first order rate constants for the reactions were obtained from the plots of logarithm of absorbance difference against time , i.e $\ln (A_t - A_\infty)$ where A_t is the absorbance at time t and A_∞ is absorbance at infinity.

3.7.1 Determination of wavelength of maximum absorption (λ_{max}) of formaldehyde and plasma albumin

The λ_{max} of formaldehyde, plasma albumin and formaldehyde- plasma albumin adduct were established using a UV/Visible spectrophotometer. The absorbance of standard solutions of formaldehyde (0.27×10^{-1} mol dm^{-3} and plasma albumin (0.75×10^{-3} mol dm^{-3}) and their mixtures were measured at different wavelengths (nm) within the range of (199 – 400) and at an interval of 10 nm. The highest peak in each case was recorded as their corresponding (λ max) of the formaldehyde, plasma albumin and plasma albumin-formaldehyde complex respectively. The molar absorptivity of formaldehyde, plasma albumin and the Plasma albumin - formaldehyde adduct were also calculated.

3. 8 Stoichiometry of the Plasma Albumin and Formaldehyde

In this study the stoichiometry of the plasma albumin and formaldehyde was determined by the spectrophotometric measurements using photometric titration, the Job's plot method and continuous variation methods. In each case, the concentration of the plasma albumin was kept constant while that of the formaldehyde was varied at constant conditions

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3.8.1 Photometric titration

The titration was carried out by measuring 2.5cm^3 of the plasma albumin in a 100 cm^3 beaker and titrating against the 0.053 mol dm^{-3} of formaldehyde from a burette . The absorbance measurements were taken at every 0.5cm^3 increment of the formaldehyde solution from a spectrophotometer. The measurements were made until the reactions indicated little or no change in absorbance after two or more readings. Triplicate measurements were made and from the end point data the molarity of the plasma albumin was calculated and a plot of absorbance versus the mole ratio of the reactants was read as the stoichiometry of the isolated plasma albumin with formaldehyde.

3.8.2 The Job's method

The Job's Method also called the method of continuous variation was employed at constant conditions, the ionic strength of the reactants μ, was kept constant at 0.5moldm3 (NaCl), and at constant temperature of 370C. Then 0 – 10 mL of formaldehyde solutions was accurately transferred into a of 50 mL measuring flask. To each flask 5mL of sodium acetate buffer was added followed by 2.0 mL of hydroxylamine hydrochloride. Also 10 - 0 mL of the plasma albumin solution was added into each of the above flasks as in first step respectively bringing the volume of mixture to 17mL. The content of each of flask was then diluted to the mark with distilled water, the solution allowed to stand for 10 minutes and its absorbance measured and was recorded using water as reference at λ max 235nm.. The absorbances of the solutions were plotted against the mole fractions of formaldehyde to find the stoichiometric ratio the stoichiometry of the adduct from the resulting curve

(Huang,1982). The model plot of absorbance versus mole fraction is presented in Appendix M

3. 8.3 The mole ratio method

In this method the concentration of plasma Albumin was kept constant at 5.1 x 10^{-4} mol dm^{-3} while that of HCHO was varied between (0.27 - 0.00027) mol dm^{-3}. The ionic strength, $I^{½}$ = 0.5 mol dm^{-3} was kept constant and at constant temperature of 37^0C. For each run 2cm^3 of the plasma albumin was taken in a quartz cuvette and kept in a spectrophotometer, while 2.0 cm^3 each of the HCHO previously treated with 0.1 $moldm^{-3}$ NaOH solution was taken in a 50cm^3 beaker thoroughly mixed and was warmed in a water bath to 37^0C. Then 2cm^3 of the formaldehyde mixture was taken and quickly mixed with the plasma albumin in the quartz cuvette using a syringe and the spectrophotometer light shield closed. The increase in absorbance was recorded at intervals of five seconds and the reaction was allowed to go to completion as indicated by little or no change in absorbance. The stoichiometry was then determined from a plot of mean absorbance against the mole ratio [HCHO]/[Albumin] (Chriswell and Schilit, 1975). A model plot of absorbace versus mole ratio is as presented in Appendix N

3. 8. 4 Determination of rate and order of the reactions of plasma albumin with formaldehyde in water solution

The rate of disappearance of plasma albumin in the formation of the adduct from the reaction of formaldehyde with plasma albumin in aqueous solution was followed in situ at its maximum wavelength (235nm) in a compartment of a UV spectrophotometer . The study was conducted at temperatures of between 20 - 40^0C in water in a stoppered cuvette. All the kinetic runs were carried out in triplicates and the curves drawn using Microsoft office exel, 2007 and graphic pad software.

The kinetic measurements were carried out at constant conditions with the concentrations of the formaldehyde in excess of 0.27 x10^{-1} mol dm^{-3} while that of plasma albumin was kept constant at 0.51 x10^{-4} mol dm^{-3}. The reaction was carried out by mixing 1-2 cm^3 of the plasma albumin with 2-3 cm^3 of 0.27 x10^{-1} mol dm^{-3} HCHO and $\mathbf{I}^{½}$ = 0.5 mol dm^{-3}. Triplicate values were taken in each case to obtain the pseudo-first and second order rate kinetics .

3. 9 Reactants Concentrations Dependence Studies

The reactants concentration dependence studies was carried out at constant ionic strength, $I^{½}$ = 0.5 mol dm^{-3} constant temperature of 37.0 ^{0}C, and constant pH of 7.0$\pm$3 but at varying concentrations of the plasma albumin and the aqueous formaldehyde. The dependence of the reactions on the reactants concentrations was established from a plot of absorbance against time for selected concentration of the analyte being varied.

3.9.1 The effect of formaldehyde concentration

The effect of the aqueous formaldehyde concentration on the reaction rate interms of absorbance intensity was monitored in the range (0.067 - 0.33) x 10^{-1} mol dm^{-3} where its various concentrations were varied while keeping the concentration of the plasma albumin at 0.51x10^{-4} mol dm^{-3} and all the other conditions constant. The dependence of the reaction of plasma albumin on the concentrations of the various aqueous formaldehyde was established from a plot of ln (A_t - A_∞) against time at each of the varied formaldehyde concentrations. Triplicate values were taken in each case to obtain the pseudo-first and second order rate kinetics.

3.9.2 The effect of plasma albumin concentration

The effect of the concentration of plasma albumin on the reaction was monitored in the range 0.75 – 0.51 x 10^{-3} mol dm^{-3} where it was varied while keeping the concentration of the aqueous formaldehyde and all the other conditions constant. The dependence of the reaction on the concentrations of plasma albumin was then established from a plot of ln (A_t - A_∞) against time at each of the concentrations of plasma albumin

3.10 Effect of Hydrogen Ion Concentration on the Reaction Rate

The effect of changes in the hydrogen ion concentration on the reaction was investigated by keeping the concentration of the reactants constant while varying the hydrogen ion concentration in the range 0.1- 1.0 x 10^{-2} mol dm^{-3} in water solution and ethanol-water mixtures. The range of hydrogen ion concentration was narrow because reactions of formaldehyde are reported to be hydrongen ion concentration sensitive but the acid was found to be at such range. The ionic strength of the reaction medium was maintained at $I^{½}$ = 0.5 mol dm^{-3} in both water and ethanol-water media. The order of reaction with respect to hydrogen ion concentration was obtained as the slope of the plot of $logk_{obs}$ versus $[H^+]$. Variation of acid dependent second order rate constants with $[H^+]$ was obtained by plotting k_2 against $[H^+]$.

The $[H^+]$ values used were calculated from the pHs using equations (3.6 - 3.8)

$$pH = \log_{10} (1/[H^+]) \quad \text{----------3.6}$$

$$\mathbf{pH = -\log_{10} [H^+] \quad ----------3.7}$$

$[H^+]$ = 10^(-pH) (Stewart, 2009) --------- 3.8

3.11 Temperature Dependence Studies

The effects of temperature on the rate constants of the reaction of plasma albumin with formaldehyde in water solution and ethanol-water mixtures was investigated under seven temperatures from 20 - 45^0C. The temperature dependence of the reactions was carried out at constant reactants conditions: 0.5 mol dm^{-3} and pH 7.0 ±3, varying (1-25%) ethanol-water mixtures as well as in water solution. The effect of the temperature variations on the reaction of plasma albumin with formaldehyde was then established from a plot of ln (A_t - A_∞) against time at each of the varied temperatures. Triplicate values were taken in each case to obtain the

pseudo first and second order rate kinetics. The energy of activation was determined from the plot of lnk against 1/T according to the Arrhenius equation Also using the Erying plots, that is $\ln(k_2/T)$ vs 1/T as a function of different temperature rate constants were plotted and the activation parameters were calculated from the relation (3.9) (Keush,2003; Asadi *et al.*, 2010).

$$\ln (k_2 / T) = - \Delta H^{\#} /RT + \Delta S^{\#}R + \ln kB/ h c^0 \quad \text{----------3.9}$$

while the activation energy was calculated by using the Arrhenius expression given in (3.10). The values of the activation energy were determined and the results recorded.

$$\mathbf{Ea = - \Delta H^{\#} + RT \text{----------3.10}}$$

3. 12 Effect of Ionic Strength on the Rate in the Reaction Medium

The effect of ionic strength ($I^{½}$) on the rate of reaction in water solution and ethanol-water mixtures was investigated over the range of (0.1 – 0.9) $moldm^{-3}$ [NaCl] The various ionic strength concentrations were varied during the run while other reaction conditions were kept constant. The ionc strength dependence on second order rate constants of the reaction was obtained by plotting k_2 against ($I^{½}$).

3.13 Effect of Added Ions on the Reaction Rate

The effect of added ions on the reaction rate was observed by addition of 0.05 - 1.0 mol dm^{-3} of the ions (CO_3^{2-}, NO_3^- , Ca^{2+}, Fe^{3+} and Zn^{2+}) , while the concentration of the formaldehyde and plasma albumin , temperature and ionic strength were kept constant and the dependence of the added counter cations and anions on the reaction rate was obtained by plotting k_2 against the concentrations of the various added ions.

3.14 The Effect of Varying Dielectric Constants on the Reaction Rate at Different Temperatures

The reaction was studied at constant conditions at various dielectric constants and temperatures of 1%, 5%, 15%, 20% and 20^0C 25^0C, 30^0C and 37^0C respectively. The rate constant as a function of different permittivity in ethanol-water mixtures was determined at constant conditions. The dielectric constant of the reaction medium was altered by the addition of the varying amounts of ethanol – water mixtures (1- 25% v/v) in a cuvette and the absorption of 0.51 $x10^{-4}$ mol dm^{-3} plasma albumin and 0.27 $x10^{-1}$ formaldehyde- plasma mixtures at 235 nm were measured. Rate was measured in (1% to 25% v/v) ethanol- water mixtures. The values of dielectric constants for different aqueous Ethanol- mixtures were taken from literature (Ik-Hwan *et al*., 2007). Triplicate values were taken in each case to obtain the pseudo-first and second order rate kinetics from a plot of ln (A_t - A_∞) versus time. The Erying plots, that is ln (k_2/T) versus 1/T for different dielectric constant were plotted and the activation parameters were calculated from the relation (3.11).

$$\ln (k_2 / T) = - \Delta H^{\#} /RT + \Delta S^{\#\#} R + \ln kB/ h c^0 \quad \text{----------3.11}$$

while the activation energy was calculated by using the plot of lnk against 1/T according to the Arrhenius equation (3.12).

$$\mathbf{Ea = - \Delta H^{\#} + RT \text{----------3.12}}$$

The value of the activation energy were determined as a function of dielectric constant of medium and the results recorded. Also the values of the second order rate constant lnk_2 were plotted against reciprocal dielectric constant to obtain the relation of the change in dielectric constant and the rate of the reaction between plasma Albumin and Formaldehyde.

3.15 Spectrophotometric Determinations of the pKa of Plasma Albumin

The associated changes in the UV-visible spectrum of a compound upon ionisation can be used in order to establish its acidity constant (pKa). Accordig to Tong (2003), it is

usually necessary to compare the spectrum of a free substrate (plasma albumin) to those of a closely related derivative lacking the properties of the original group. In this present case we compared the absorption of 51 $x10^{-4}$ mol dm^{-3} plasma albumin to that of its adduct with 0.27 $x10^{-2}$ mol dm^{-3} formaldehyde absorption at pH range of 5.3- 8.7.

The Spectrophotometric determinations of the pKa of plasma albumin was done using a procedure similar to that of Polgar (1974) where differences in the absorption of the 0. 51 $x10^{-4}$ mol dm^{-3} plasma albumin and its Plasma albumin-formaldehyde adduct at 235 nm were measured at varying pH and the molar absorptivity calculated . A solution of 0.5 mol dm^{-3} NaCl and a self buffering solution of NaOH – HCl at a temperature of 37^0C and varying pH 5.3 – 8.8 in varying ethanol-water mixtures of different permittivities of 78 – 64.9, were used. The absorbance of both plasma albumin and the formaldehyde – plasma albumin adduct were measured separately at the selected pH in a spectrophotometer titration and the values recorded. Triplicate measurements were made at each selected pH and the mean absorbance value taken. The difference in absorbance was obtained and the molar absorptivity values calculated. The pKa values were obtained from plots of absorptivity $\Delta\varepsilon_{235}$ versus pH and $1/\Delta\varepsilon_{235}$ versus $[H^+]$ from the relation $1/\Delta\varepsilon_{235} = [H^+]/ \varepsilon_{235} . Ka + 1/\varepsilon_{235.}$ The Bronsted constant was obtained from the slopes of the plots of lnk_2 against the pKa values (Tong, 2003).

CHAPTER FOUR

4.0 RESULTS

4.1 Analysis of Formaldehyde Levels in Human Urine, Milk and Food-Related Samples

Tables 4.1a - 4.1c and Figure 4.1 show the formaldehyde levels and the correlational analyses results obtained from human urine, milk and the food related samples studied using acetylacetone and acetoacetanilide reagents which were determined at low temperatures based on the Hantzsch reaction. The reaction involving acetoacetanilide was measured at 365 – 368 nm at room temperature while the detection involving the acetylacetone was measured at 400 – 415 nm (Li *et al.*, 2007). The results show appreciable but low levels of formaldehyde in g/Kg in all the samples analysed relative to the WHO (1989) toxic limits for foods and non foods samples in the two reagents. The results also show that both reagents used gave comparable values of formaldehyde in the samples analysed as shown in the correlational analyses result in Table 4.1c and Figure 4.1 respectively.

4.2 Analysis of Formaldehyde Levels in Cosmetics and Non – Food Materials

Tables 4.2a and 4.2b show the formaldehyde levels obtained from the cosmetic materials and non-food materials studied using acetoacetanilide and acetylacetone reagents (both ß-ketones). These were also determined at room temperature based on the Hantzsch reaction. The detection reaction involving the cyclization between acetoacetinalide and formaldehyde in the presence of ammonia that was derived from ammonium citrate at 365-368 nm at room temperature resulted in the formatioon 1, 4 dihydropyridine dicarboxylate adduct while the detection reaction between acetylacetone and formaldehyde in the presence of ammonia derived from ammonium citrate that was measured at 400 - 415 nm (Li *etal.,* 2007) formed a 3,5-diaacetyl 1,4-dihydrolutidine. The results show appreciable but low levels of formaldehyde in g/Kg in the cosmetics and non-food samples relative to the world Health Organization (WHO) toxic limits for foods and non foods samples in the two reagents. The results also show that both reagents used gave comparable values of formaldehyde in the samples analysed as shown in correlational analyse (Table 4.3 and Figure 4.2).

4.3 Characterization of Isolated Plasma Albumin and Prepared Adduct

Table 4.4 show the results of percentage recovery, optical rotation, solubility in water, denaturation test and %protein content of the isolated plasma albumin sample. The results showed a protein content of 63.67%, with a very poor percentage recovery of 1.36 % (2.4g). Also the isolated albumin was observed to be slightly soluble in water and ethanol and was slightly denatured with a specific rotation [α] 59832 value of 8.0 at pH 7.4. In spite of low percentage recovery and low solubility in water the quality of plasma albumin was found to be active and pure in its native state (Michael, 1968). Also the method allowed for isolation and recovery of native state plasma albumin from

simple and available reagents and equipment without resulting to the expensive and time consuming chromatographic techniques.

Table 4.1a: Formaldehyde levels in human urine, milk and food-related samples by acetylacetone reagent

Sample	Concentration (Mol dm^{-3})	Concentration (g/kg)
Human urine composite	1.55 (±0.02)	46.55
Human milk (composite	0.67 (±0.01)	18.01
Raw Cow Milk	0.50 (±0.01)	13.21
Peak Milk (P)	0.44 (±0.01)	11.11
Nunu milk (P)	0.34 (±0.001)	5.11
Dry fish (local)	0.63 (±0.01)	18.32
Fried fish (Mackerel)	0.71 (±0.01)	20.42
Frozen fish (Mackerel)	0.22 (±0.01)	6.61
Smoked fish (mackerel)	0.60 (±0.02)	18.02
Fresh fish (local)	1.44 (±0.02)	42.24

Table 4.1b: Formaldehyde levels in food samples, human milk and urine using acetoacetanilide reagent

Sample	Concentration (Mol dm^{-3})	Concentration (g/kg)
Human urine composite	1.61 (±0.02)	48.30
Human milk (composite	0.63 (±0.01)	19.01
Raw Cow Milk	0.50 (±0.01)	15.11
Peak Milk (P)	0.44 (±0.01)	13.1
Nunu milk (P)	0.34 (±0.001)	10.31
Dry fish (local)	0.63 (±0.01)	18.87
Fried fish (Mackerel)	0.71 (±0.01	21.20
Frozen fish (Mackerel)	0.28 (±0.01)	8.52
Smoked fish (mackerel)	0.63 (±0.02	18.80
Fresh fish (local)	1.51 (±0.02)	45.44

Table 4.1c: Correlational analysis of formaldehyde levels in food-related samples, human milk and urine using acetylacetone and acetoacetanilide reagents

Parameter	Acetylacetone method	Acetoacetanilide method
Number of XY Pairs	10	10
Pearson r	-0.04040	-0.03713
90% confidence interval	-0.5798 to 0.5237	-0.5777 to 0.5261
P value (two-tailed)	0.9118	0.9189
P value summary	Ns	Ns
Is the correlation significant?(alpha=0.05	No	No
R squared	0.001632	0.001378

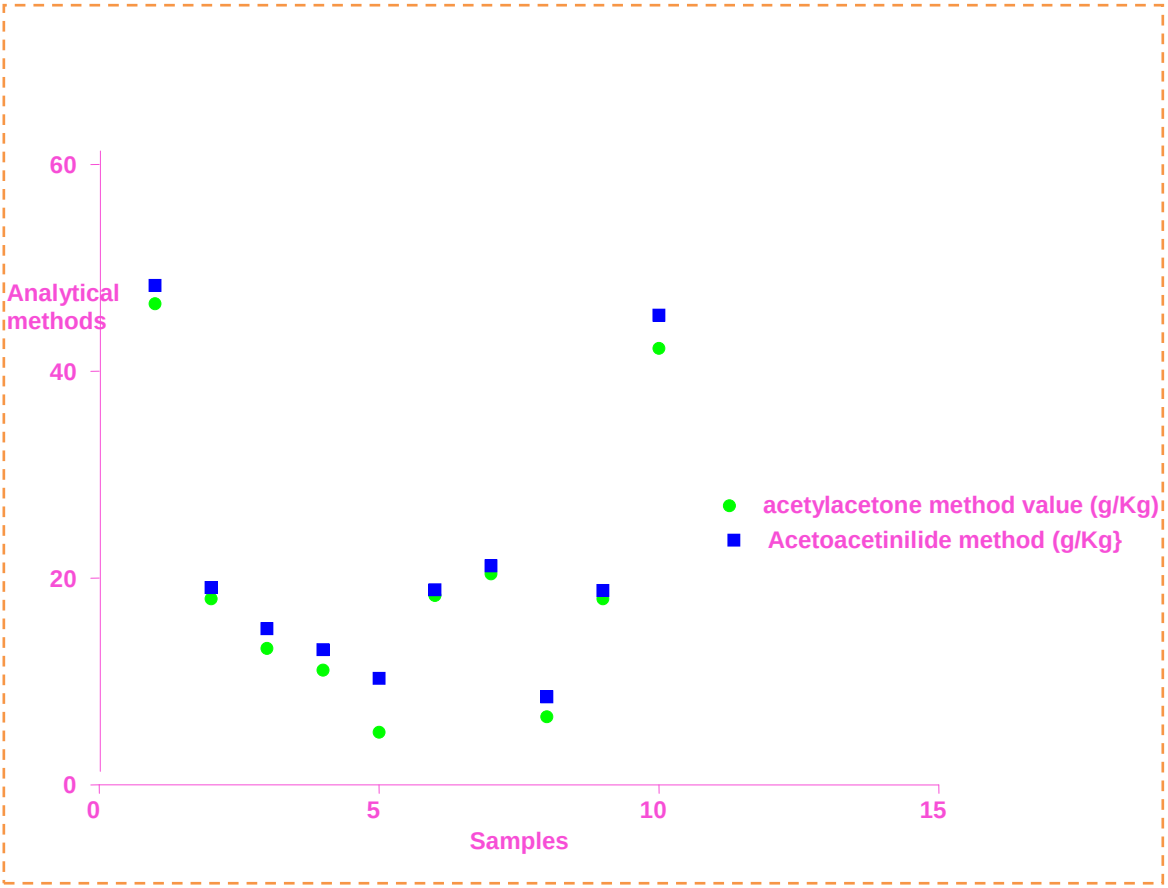


Figure 4.1: Correlation analysis for formaldehyde levels in food samples, human milk and urine using acetylacetone and acetoacetanilide methods

Table 4.2a: Formaldehyde levels in cosmetics and non-food materials using acetylacetone reagent

Sample	Concentration (Mol/d^3)	Concentration g/kg
Napkin1	0.4 (±0.01)	12.01
Napkin 2	0.3 (±0.02)	09.01
Napkin 3	0.28 (±0.01)	08.41
Lipstic 1	0.32 (±0.020)	9.61
Lipstic 2	0.54 (±0.006)	16.22
Lipstic 3	0.48 (±0.001)	14.41
Petals 1	3.16 (±0.02)	94.89
Petals 2	3.28 (±0.03)	98.50
Petals 3	1.42 (±0.02)	42.64
Toilet tissue1	0.54 (±0.02)	16.22
Toilet tissue 2	0.47 (±0.01)	14.11
Toilet tissue3	0.40 (±0.02)	12.01
Growers 1	0.53 (±0.006)	15.92
Growers 2	0.62 (±0.02)	18.62
Layers	0.25 (±0.006)	7.51
Paint 1	0.47 (±0.01)	14.11
Paint2	0.53 (±0.006)	15.92
Paint 3	0.27 (±0.01)	8.00

Table 4.2b: Formaldehyde levels in cosmetics and non-food materials using acetoacetanilide reagent

Sample	Concentration (mol/ dm^3)	Concentration g/kg

Napkin1	0.44(±0.01)	13.30
Napkin 2	0.41(±0.02)	12.21
Napkin 3	0.34(±0.01)	10.21
Lipstic 1	0.33 (±0.020)	9.99
Lipstic 2	0.58(±0.006)	17.52
Lipstic 3	0.56(±0.001)	16.71
Petals 1	3.21 (±0.02)	96.44
Petals 2	3.30 (±0.03)	99.02
Petals 3	1.48 (±0.02)	44.45
Toilet tissue1	0.57 (±0.02)	17.12
Toilet tissue 2	0.49 (±0.01)	14.81
Toilet tissue3	0.42 (±0.02	12.61
Growers 1	0.55 (±0.006)	16.52
Growers 2	0.63 (±0.02)	18.88
Layers	0.43 (±0.006)	12.83
Paint 1	3.21 (±0.02)	14.70
Paint2	0.54 (±0.006)	16.25
Paint 3	0.38 (±0.01)	11.55

Table 4.3: Correlational analysis of formaldehyde levels in (cosmetics) non-food materials using acetylacetone and acetoacetanilide reagents

Number of XY Pairs	**18**	**18**
Pearson r	-0.1141	-0.1144
95% confidence interval	-0.5517 to 0.3727	-0.5519 to 0.3725
P value (two-tailed)	0.6520	0.6514
P value summary	Ns	Ns
Is the correlation significant? (alpha=0.05)	No	No
R squared	0.01303	0.01308

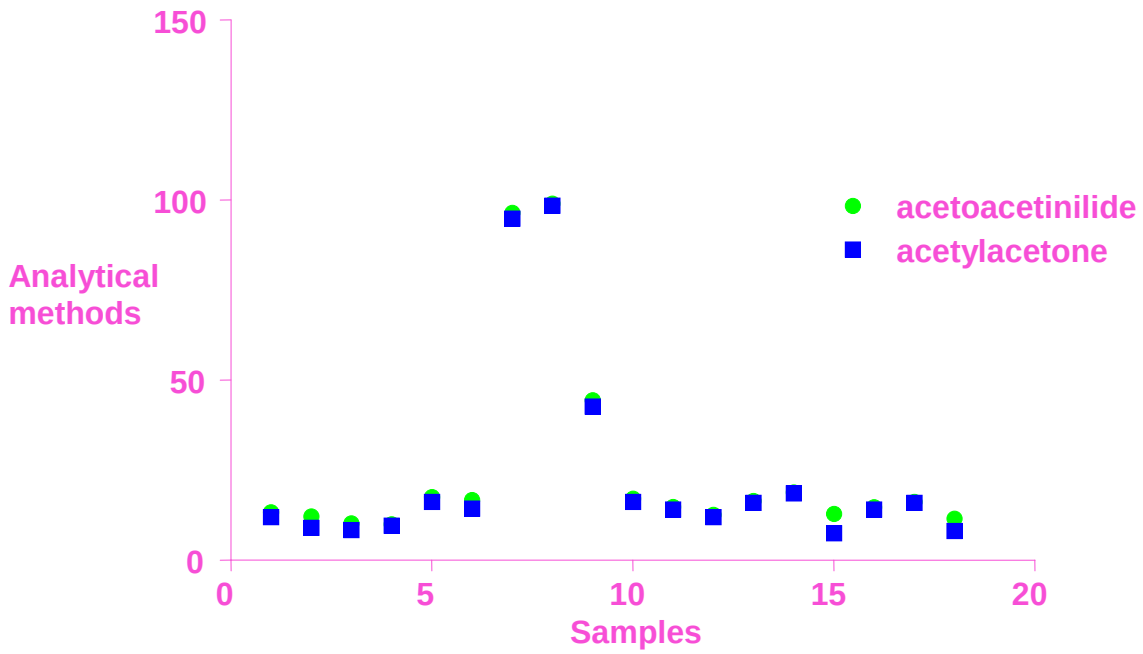


Figure 4.2: Correlation analysis chart of formaldehyde values obtained from cosmetics and non-food samples using acetylacetone and acetoacetanilide reagents.

Table 4.4: % Protein, solubility, denaturation test and % recovery of the isolated plasma albumin

Parameters	Results
% Protein	63.67
Optical rotation, $[\alpha]_D^t$	08.00
Solubility in water	Dissolved gradually in water
Denaturation test	No precipitate or turbidity when dissolved in water

4.4 Ultra Violet Spectral Analyses of 1, 4 dihydropyridine

Figure 4.3 shows the 1,4-dihydropyridine adduct that was prepared and characterized for its properties in this study. It was found to absorb the ultra violet light strongly at three wavelengths (λ_{max}) 250, 255 and 410 nm with absorption coefficients (ϵ) of 1645, 1812 and 1483 L mol^{-1} cm^{-1} respectively.

4.5 Melting Point, Antimicrobial Susceptibility and Infrared Spectroscopy Analyses of 1, 4-dihydropyridine Adduct

Tables 4.5, 4.6 and Figures 4.4 and 4.5 show the results of melting point, antimicrobial susceptibility and infrared spectroscopy analyses for the prepared 1,4-dihydropyridine adduct based on the method of Hantzsch reaction (Li *et al.,* (2007). The result of the melting point analysis shows that theadduct had a melting point range of (179.8 - 180.4^{0}C). This indicated that the adduct is labile Schiff base with similar characteristics with those found in the literature which were referred to gylcated products (Povey *et al.,* 2008).

The result of microbial activity analyses show that the 1,4-dihydropyridine adduct have some antimicrobial effect on some micro-organisms such *as Bacillus subtilis* zone of inhibition ,12.0 mm, *Proteum mirabilis* zone of inhibition , 16.0 mm *and Canidad albicans* zone of inhibition , 14.0 mm The adduct have no effect on some micro organisms such fungi /algae like *Escherichia coli* and *Staphylococcus aureus.* This shows that the adduct has no active antimicrobial activity over fungi and algae.. This property was reported to be typical of lectin action mechanisms, and it was observed that the 1.4-dihydropyridine adduct had the specialized glyco-protein properties (Peumans and VanDamme, 1995) as shown in Figure 4.5

The IR analysis showed the functional groups present in the adduct. The band at 2900.76 cm^{-1} was assigned to aldehyde C-H stretch and the one at 3845.5 cm^{-1} was

found to be (O-H) stretch for water of hydration. The sharp band at 2356.87 was found to be due to methylene bridge CH_2 stretch in the adduct while the bands at 1647.2, 1517.4 and 1032.63 cm^{-1} were assigned to N-H amines band and C-N amines stretch respectively. The bands were assigned based on the principle of (Hsu,1997).

4.6 Kinetic Studies

4.6.1 Wavelengths of maximum absorption (λ_{max}) of formaldehyde, plasma albumin and formaldehyde - plasma albumin adduct

Figure 4.6 - 4.8 show the absorptions and wavelengths of maximum absorptions as well as the molar absorptivity values of formaldehyde, plasma albumin and Formaldehyde-plasma albumin adduct. Both Formaldehyde and Plasma albumin were found to absorb maximally very closely within the same uv region at a λ_{max} of 210 nm. Plasma albumin however was also found to absorbed more widely within the range of 198-250 nm and at other wavelengths (350 and 400 nm) respectively. Interestingly, the adduct formed between formaldehyde and plasma albumin was observed to absorb maximally throughout the wavelength range of 198- 400 nm that was investigated with a λ_{max} 240 nm as given in Figure, 4.8.

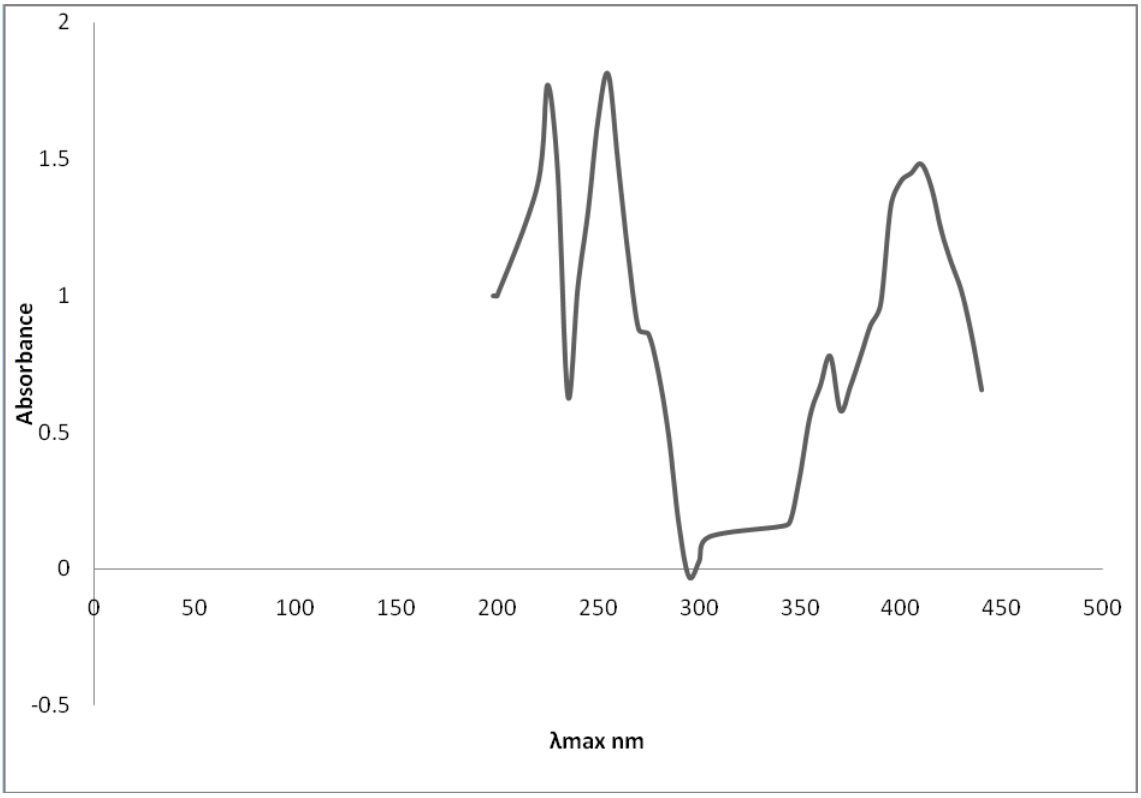


Figure 4.3: The three wavelengths of maximum absorptions λ_{max} exhibited by the synthesized 1, 4-dihydropyridine adduct at 250, 255 and 410 nm

Peak finding results for: amino acid
Frequency: 406.75 - 4000.00, threshold: 88.086, sensitivity: 50.00
Peak finding result table:

Peak#	1	2	3	4	5	6	7	8
Position	3850.70	3736.73	3617.47	2357.04	1739.68	1547.01	1032.33	670.73
Height	69.126	67.360	68.292	52.241	74.880	75.780	78.714	83.211

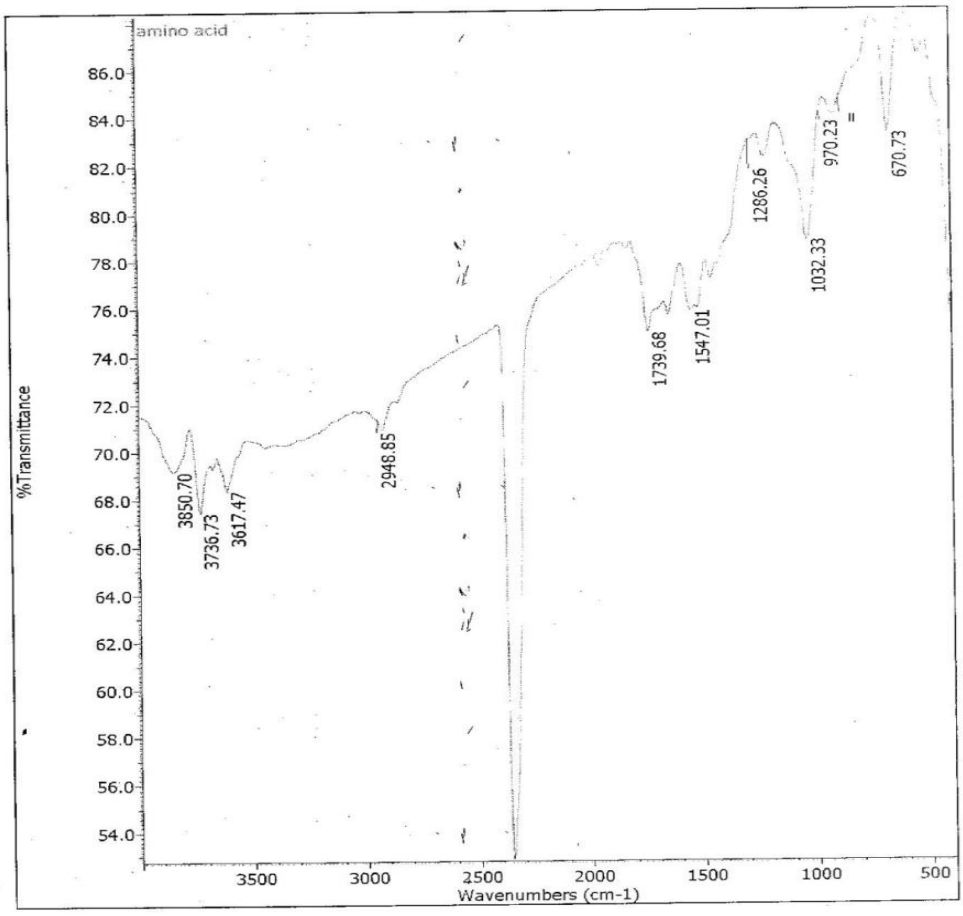


Figure 4.4: The percentage transmittance bands and intensities in wave numbers (cm^{-1}) of 1, 4-dihydropyridine adduc

GLYCOPROTEIN

Figure 4.4: Lectin Mechanisms of the Synthesized1,4-Dihydropyridine Adduct

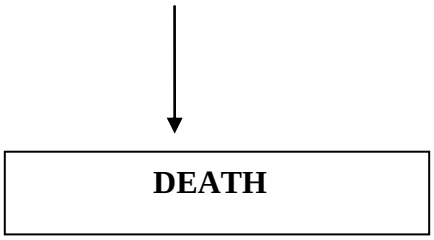


Figure 4.5: The glycoprotein mechanism of the prepared 1, 4-dihydropyridine adduct

Table 4.5: Melting point range of 1, 4-dihydropyridine adduct

Initial melting point ^{0}C	Final melting point ^{0}C

179.5	180.2
180.0	180.4
180.0	180.5
Mean sample melting point range	179.8 -180.4

Table 4.6: Antimicrobial activity of 1, 4-dihydropyridine adduct

Test organisms	**Zone of inhibition (mm)**

Bacillus subtilis	12.0
Proteum mirabilis	16.0
Canida albicans	14.0
Escherichia coli	0.00
Staphylococcus aureus	0.00

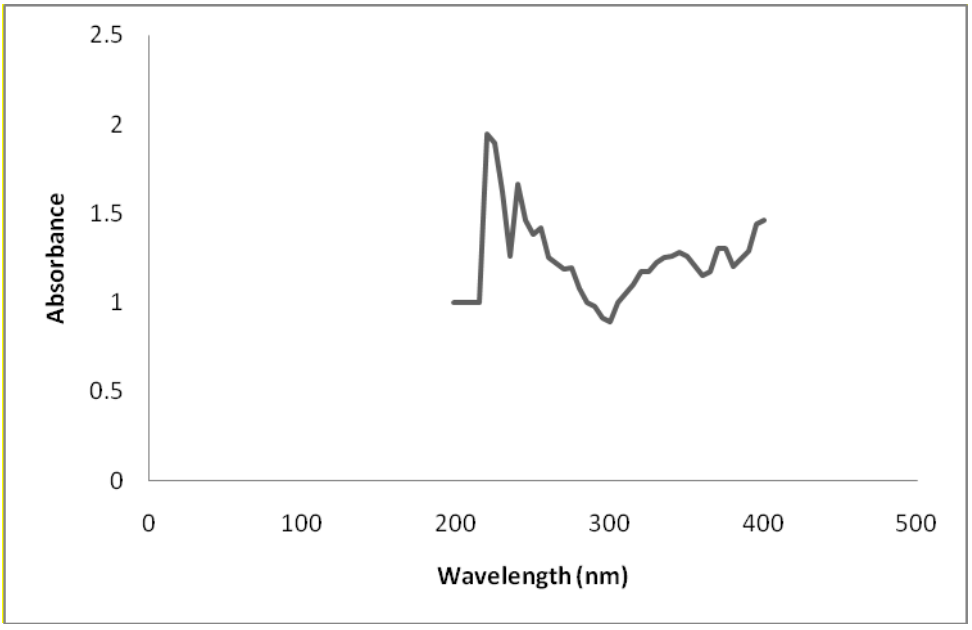


Figure 4.6: Wavelength of maximum absorption of plasma albumin

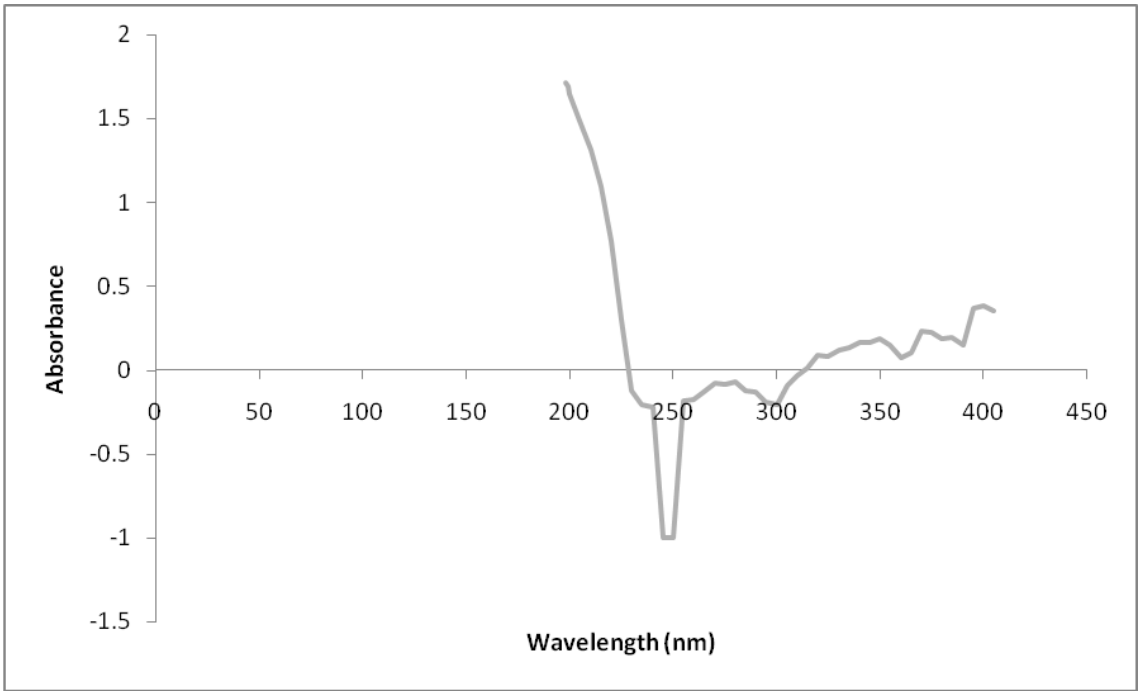


Figure 4.7: Wavelength of maximum absorption of formaldehyde

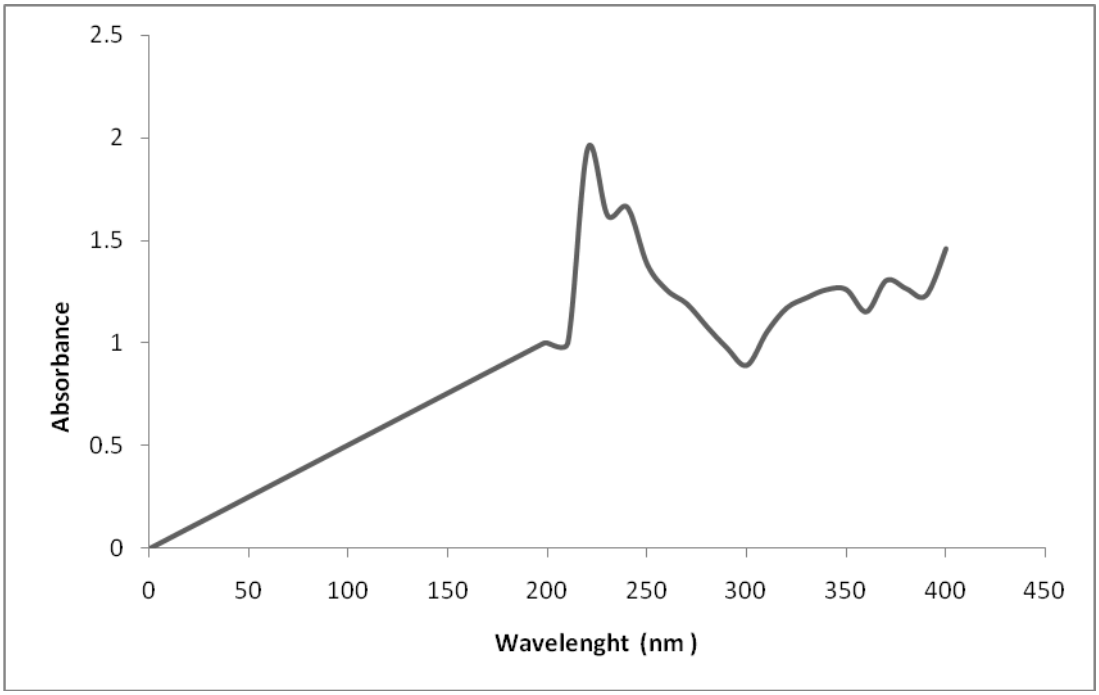


Figure 4.8: Wavelength of maximum absorption of formaldehyde - plasma albumin adduct

4.6.2 Stoichiometry

Tables 4.7 and 4.8 show the absorbencies with their corresponding mole fractions values obtained using the mole ratio and the continuous variation methods. The results of the stoichiometric analyses showed that one mole of plasma albumin is consumed by two moles of formaldehyde. The curves form which the stoichiometry was determined is presented in Figures 4.9 and 4.10. From the plot obtained, tangents drawn on both sides of the maximum and perpendicular line from the middle point to the x- axes represented the mole fraction of the plot. From figure 4.9 a value of 0.26 was observed, this value is less than 0.5 which gives the mole ratio of the plasma albumin to formaldehyde as 2: 1 (Huang, 1982).

On the basis of these results, the over all equation for the reaction in water solution can be represented as follows:

$$H_2C(OH)_2 + 2PABNH_2 \rightarrow \ PAN(HCH_2\ OH)_2 - - - -4.1$$

4.6.3 Order with respect to reactants in water

Table 4.9 show the results of the pseudo-first order and second order rate constants for the plasma albumin and formaldehyde reaction in water. The results show a mean second order rate constant of 0.099(±0.034) dm^3 mol^{-1} s^{-1} from the varying formaldehyde concentrations of between 0.27 x 10^{-1} - 1.0 x 10^{-1} mol dm^{-3} and a constant plasma albumin concentration of 0.51x10^{-4} mol dm^{-3} at the given constant conditions. All the measurements obeyed pseudo first order conditions and the second order rate constant of the reaction in water was obtained from the slope of the combined plots of of ln (A_t - A_∞) logk_1 versus time as lso presented in Appendices (G – L.).

Table 4.7: Stoichiometry for the reaction of plasma albumin with formaldehyde at human body temperature, [formaldehyde] = 2.7- 10 x 10^{-1} mol dm^{-3}, [albumin] =

0.51×10^{-3} moldm^{-3}, $I^{1/2}$ = 0.5 mol dm^{-3}, ph= 6.8, λ_{max} = 235nm and T= 37.0 ^{0}C by photometric titration method

Absorbance	Mole fraction
1.853	0.11
1.766	0.23
1.133	2.05
1.208	2.26
1.224	2.37
1.286	2.65
1.323	3.01
1.373	3.47
1.445	4.10
1.500	5.01
1.602	6.44
1.678	7.52

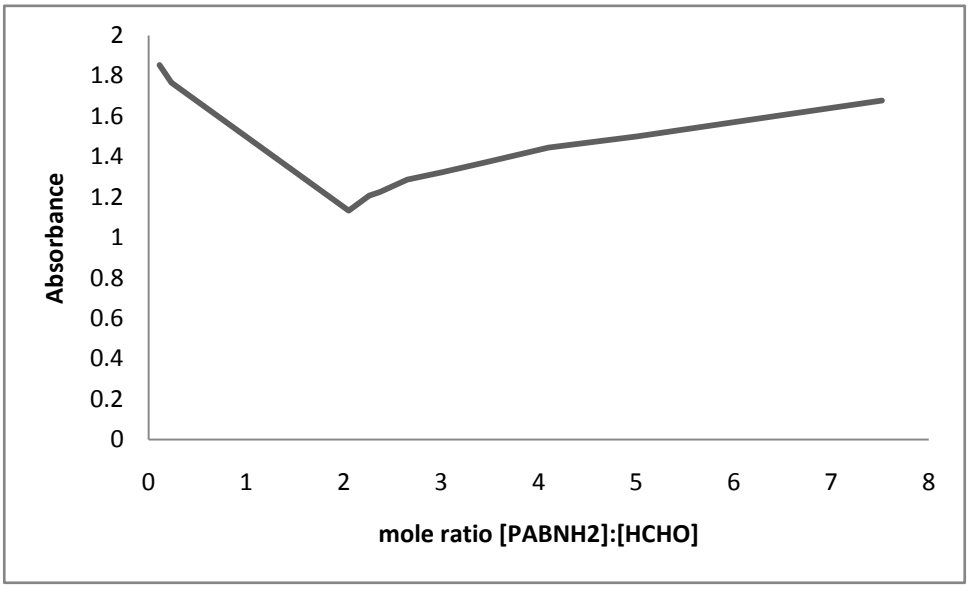


Figure 4.9: Plot of absorbance versus mole ratio for the reaction of plasma albumin with formaldehyde at human body temperature, [formaldehyde] =2.7-10) x10^{-1}mol dm^{-3}, [albumin] = 0.51x10^{-3} moldm^{-3}, I ^{½} = 0.5 mol dm^{-3}, pH= 6.8, λ_{max} = 235 nm and T= 37.0 ^{0}C

Table 4.8: Stoichiometry for the reaction of plasma albumin with formaldehyde at human body temperature, [formaldehyde] = 2.7 - x 10^{-1} mol dm^{-3}, [albumin] =

$0.51x\ 10^{-3}$ mol dm^{-3}, $I^{½}$ = 0.5 $moldm^{-3}$, pH = 6.8, λ_{max} = 235 nm and T= $37.^{0}$C by continous variation method

Absorbance	Mole Fraction
0.1	0.792
0.2	1.547
0.3	1.383
0.4	1.00
0.5	1.15

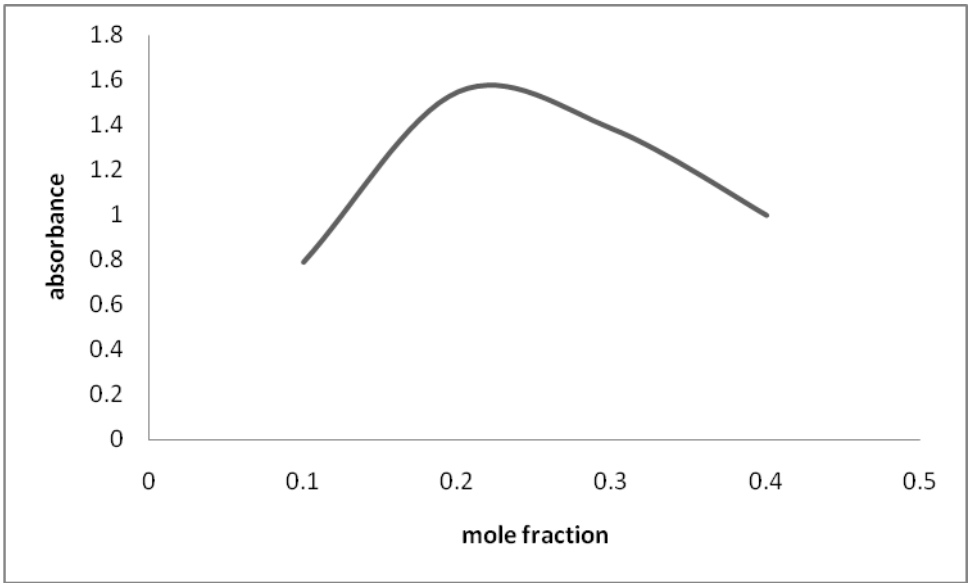


Figure 4.10: Plot of absorbance versus mole fraction for the reaction of plasma albumin with formaldehyde at human body temperature, [formaldehyde]= 2.7 - 10) x 10^{-1}mol dm^{-3}, [albumin] = 0.51x 10^{-3} mol dm^{-3} , $I^{½}$ = 0.5 mol dm^{-3}, pH = 6.8, λ_{max}= 235 and t= 37.0 ^{0}C

From the data. the mean value of the slopes of the plots (Figure 4.11) was obtained as 0.853 ± 0.21 and the second order rate constants for the various concentrations as

determined from the relation k_1 /[HCHO] to obtain a mean value 0.099 (±0.034) mol^{-1} dm^3s^{-1} the various rate constant values are as presented in table 4.9. Based on the above, the rate equation for the reaction of plasma albumin with varying concentrations of formaldehyde in water solution can be represented by equation (4.2) below.

$$\frac{-d[H_2C(OH)_2]}{dt} k_2[H_2C(OH)_2][PABNH_2] - - - - - - - - - 4.2$$

k_2 = second order rate constant, the second order rate constant,calculated from k_1 /[HCHO] were fairly constant with an average value of k_2 = 0.099 (±0.034) dm^3 mol^{-1} s^{-1}

4.6.4 Order of reaction with respect to reactants in ethanol-water- mixtures

The results of the pseudo-first order and second order rate constants for the plasma albumin and formaldehyde reaction in ethanol-water mixtures in are as presented in Tables 4.10 and Figures 4.11. The Pseudo first 0rder rate constants of the reaction for the various formaldehyde concentrations in ethanol-water mixtures was also obtained from reaation of slope of the of ln (A_t - A_∞) versus time (sec). The mean slope obtained in ethanol-water mixtures was 0.89556 (±0.0). The mean second order rate constants (0.06 ±0.028) dm^3 mol^{-1} s^{-1} as determined from k_1/[HCHO] from the varying formaldehyde concentrations of 0.27 x 10^{-1} - 10.0 x 10^{-1} mol dm^{-3} was as presented in Table 4.11. Also a plot of Plots of logk_2 versus log [HCHO] for the Reaction of Plasma Albumin and Formaldehyde at Varying Ethanol-Water Mixtures at constant conditions followed a linear second order kinetics as presented in Figure 4.12.

Table 4.9: Pseudo - first order and second order rate constants for the reaction of plasma albumin and varying concentrations of formaldehyde in water at constant conditions and at human body temperature, [plasma albumin]= 0.51x 10,$^{-4}$ mol dm^{-3}, $I^{½}$ = 0.5 mol dm^{-3}, pH= 6.8 , λ_{max} = 235nm and T= 37.0 ^{0}C

10^{-1}[HCHO] moldm^3	10^{-6}[H^+] mol/dm^3	10^{-1}[I] mol/dm^3	k_1 s^{-1}	$k_2$mol^{-1} dm^{3}s^{-1}	logk_{obs}	log[HCHO]
2.7	1.0	5.0	0.025	0.093	-1.602	-0.569
3.0	1.0	5.0	0.029	0.095	-1.538	-0.523
6.0	1.0	5.0	0.062	0.103	-1.208	-0.222
7.0	1.0	5.0	0.072	0.102	-1.143	-0.155
8.0	1.0	5.0	0.082	0.103	-1.086	-0.097
10.0	1.0	5.0	0.102	0.102	-0.991	-0.004

Mean k_2 values **0.099 (±0.034)**

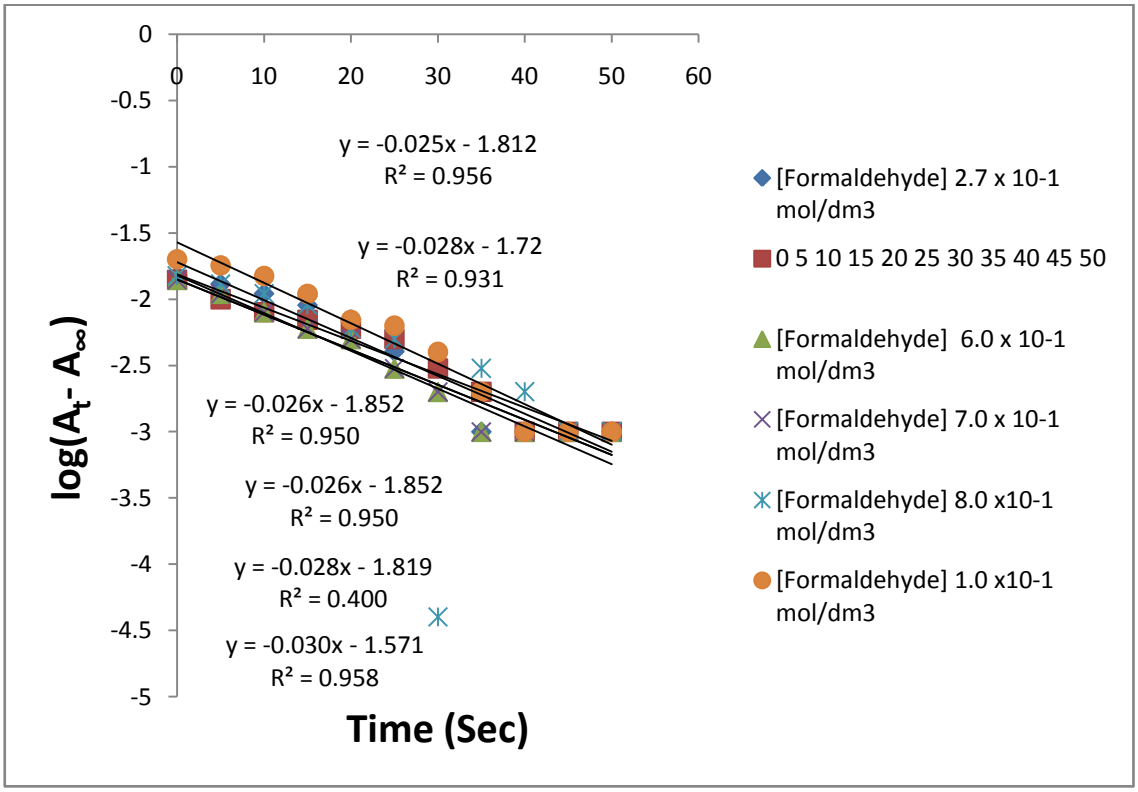


Figure 4.11: A combined pseudo - first order plots for the reaction of plasma albumin and formaldehyde in water at constant conditions of formaldehyde and plasma albumin concentrations at human body temperature, [formaldehyde] =2.7 x10^{-} moldm^{-3} – 10.0 x 10^{-3} mol dm^{-3}, [plasma albumin] = 0.51x 10^{-3} mol dm^{-3}, $I^{½}$ = 0.5 moldm^{-3}, pH = 6.8, λ_{max} = 235 nm and T= 37.0 ^{0}C

Table 4.10: Pseudo - first order and second order rate constants for the reaction of plasma albumin and formaldehyde at varying ethanol-water mixtures

(1.0-25%) and constant conditions, [formaldehyde] = (2.7 – 9.0) x10^{-1} mol dm^{-3} , [plasma albumin]= 0.51x 10^{-3} $moldm^{-3}$, $I^{½}$ = 0.5 mol dm^{-3}, $[H^+]$ = 1.0 x 10^{-6} $moldm^{-3}$ λ_{max} = 235nm and T= 37.0 ^{0}C

%[Ethwater]	10^{-1}[HCHO]	k_1 s^{-1}	k_2 $mol^{-1}dm^3$ s^{-1}	logk_2	log[HCHO]
1	2.7	0.026	0.1	-1.000	-0.569
5	3.0	0.028	0.09	-1.046	-0.523
10	6.0	0.025	0.05	-1.301	-0.222
15	7.0	0.028	0.04	-1.398	-0.155
20	8.0	0.031	0.04	-1.398	-0.097
25	9.0	0.033	0.04	-1.481	-0.046
Mean values		**0.029 (±0.03)**	**0.06 (±0.028)**		

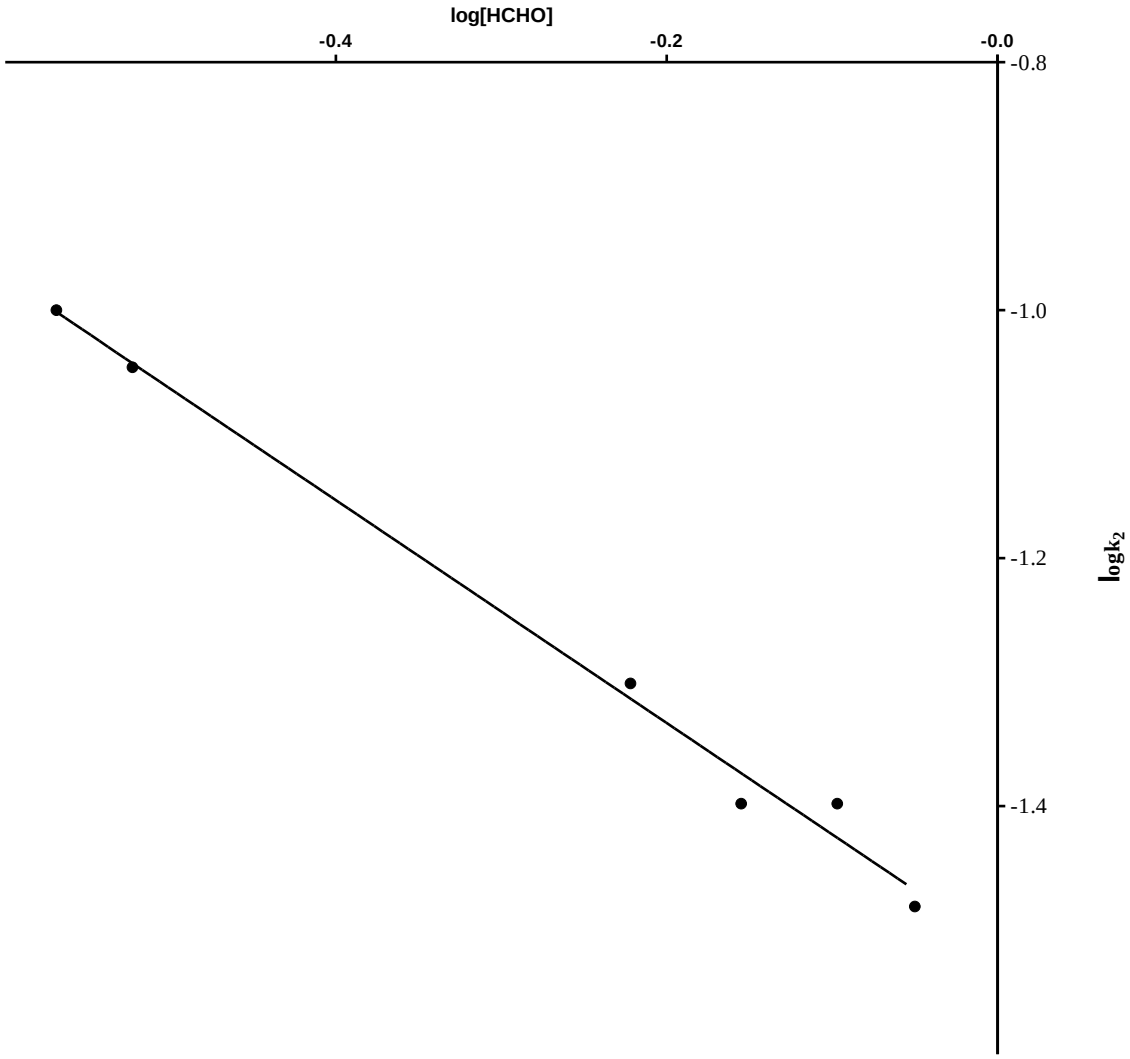


Figure 4.12 : Plots of $logk_2$ versus log [HCHO] for the reaction of plasma albumin and formaldehyde at varying ethanol-water mixtures (1.0-25%) and constant conditions, [formaldehyde] = (2.7 – 9.0) x 10^{-1} mol/ dm^3, [plasma albumin]= 0.51x 10^{-3} mol dm^{-3}, $I^{½}$= 0.5 mol dm^{-3}, $[H^+] = 10^{-6}$ $moldm^{-3}$, λ_{max} = 235nm and T= 37.0 ^{0}C

4.6.5 The effect of varying plasma albumin concentrations on the reaction rate of plasma Albumin and formaldehyde in water

Table 4.11 and Figure 4.12 present the pseudo first order and second order rate constants and the effect of varying concentrations of plasma albumin on the rate of the reaction of plasma albumin and formaldehyde as obtained from the plot of $\log k_2$ versus the concentrations of given plasma albumin. The rate constant values decreases with increase in plasma albumin concentration at the low values of formaldehyde.The value of the rate constants also clearly show that the reaction is second order reaction.

4.6.6 Ionic strength studies on the reaction of plasma Albumin with formaldehyde in water

Tables 4.12 and Figures 4.14 present the effect of ionic strength on the rate constant of the reaction between formaldehyde and plasma albumin in water solution. The results show that there is a linear rate dependence on the ionic strength of medium and the rate increased with increase in the ionic strength of the salt as obtained from the plot of $\log k_2$ versus $\log k_2/k_{obs}$ as given in figure 4.13,

4.6.7 Ionic strength studies on the reaction between plasma albumin and formaldehyde in 15% (Ethanol-water) mixture

Table 4.13 and Figure 4.15 provide the data for effect of ionic strength on the rate constant of plasma albumin and formaldehyde reaction in 15 % ethanol-water - system. The values of rate constants were found to decrease with the increase in ionic strength and concentration of ethanol, this is consistent with the Br**o**nsted theory and also in accordance to the literature (Uddin *et al,* 2006). A plot of $\log k_2$ versus $\log k_2/k_{obs}$ also showed a linear dependence of the rate decreasing with increase in the concentration of the ions in ethanol-water mixrures as presented in figure 4.14.

Table 4.11: Pseudo - first and second order rate constants values for the reaction of plasma albumin and formaldehyde in water at varying plasma albumin concentrations and constant conditions at human body temperature,

[formaldehyde] = 0.27x10^{-5} mol dm^{-3}, [Albumin]= 0.51x 10^{-3} mol dm^{-3}, I ^{½} = 0.5 mol dm^{-3}, λ_{max} = 235nm and T= 37.0 ^{0}C

[PABNH$_2$] mol dm^{-3}	**10x10^{-6}[H$^+$] mol dm^{-3}**	**k$_1$ s^{-1}**	**k$_2$dm^{3}mol^{-1} s^{-1}**	**logk$_2$**	**log[PABNH$_2$**
2.1 x10^{-2}	1.0	0.029	1.381	0.140	-1.678
3.5 x10^{-2}	1.0	0.034	0.971	-0.013	-1.456
5.1 x10^{-2}	1.0	0.035	0.686	-0.164	-1.292
7.5 x 10^{-2}	1.0	0.037	0.493	-0.307	-1.125

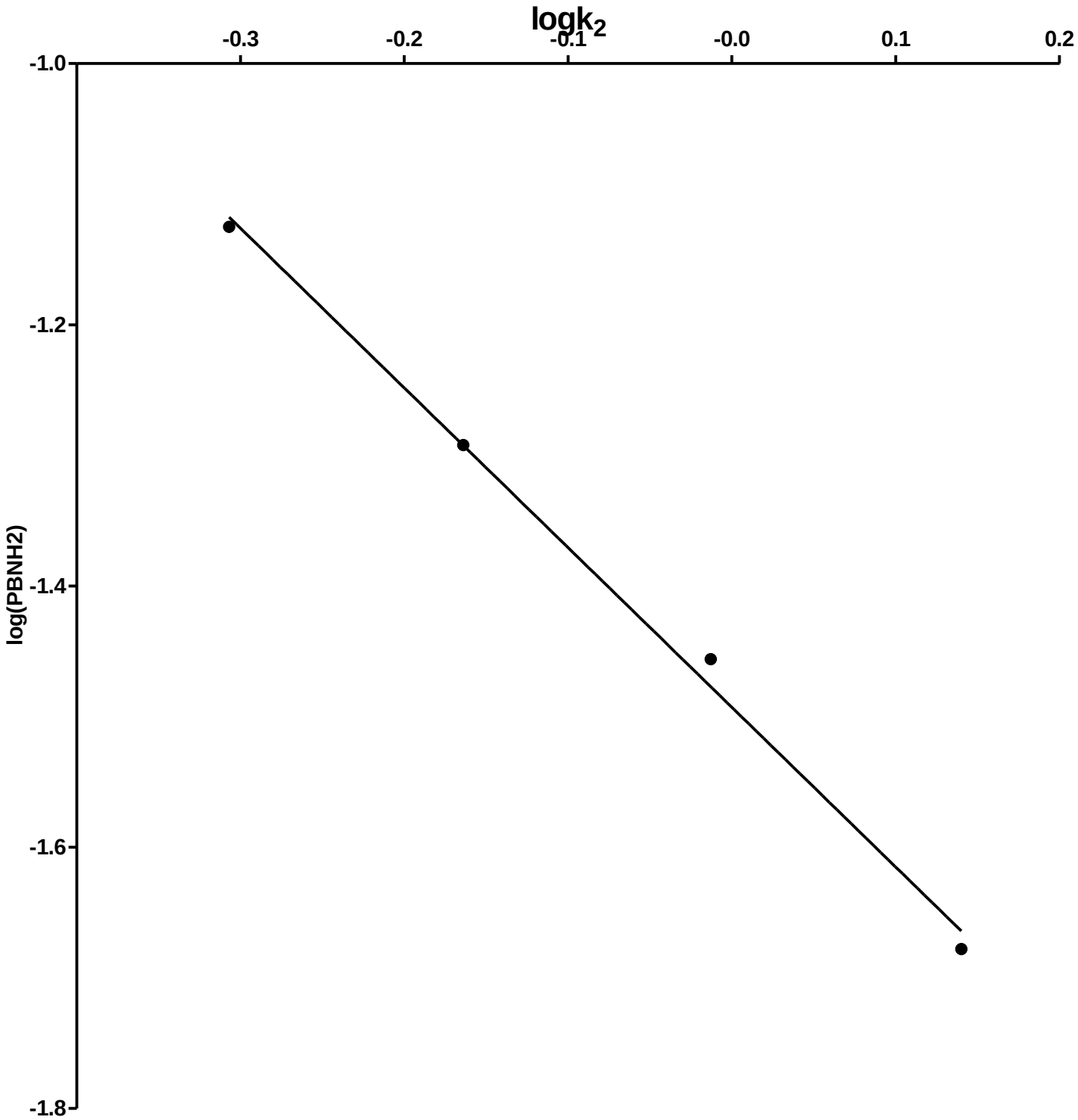


Figure 4.13: Plot of $logk_2$ versus log [$PABNH_2$] for the reaction of plasma albumin and formaldehyde in water solution at varying plasma albumin concentration and constant conditions and human body temperature, [formaldehyde]= 2.7x 10^{-4}mol dm^{-3}, [Plasma Albumin] = 2.1-7.5 x10^{-2} mol dm^{-3} , $I^{½}$ = 0.5 mol dm^{-3}, pH= 6.9, λ_{max} = 235nm and T= 37.0

Table 4.12: The effect of ionic strength on the rate constants for the reaction of plasma albumin and formaldehyde at varying ionic strength and constantconditions,[formaldehyde] = $2.7x10^{-1}$ $moldm^{-3}$, $[H^+] = 1.0 \times 10^{-6}$ mol dm^{-3} [Plsma albumin] = $0.51x\ 10^{-4}$ mol dm^{-3}, $I^{½}$ = 0.1 - 0.9 mol dm^{-3} , λ_{max} = 235 nm and T= 37.0 ^{0}C in water system

$I^{½}$	k_1	k_2	log k_2	logk_2/k_1
0.1	0.025	0.093	-1.032	-4..128
0.3	0.028	0.104	-0.971	-3,348
0.5	0.029	0.107	-0.939	-3.029
0.7	0.031	0.115	-0.983	-3.511
0.9	0.032	0.119	-0.924	-2.888

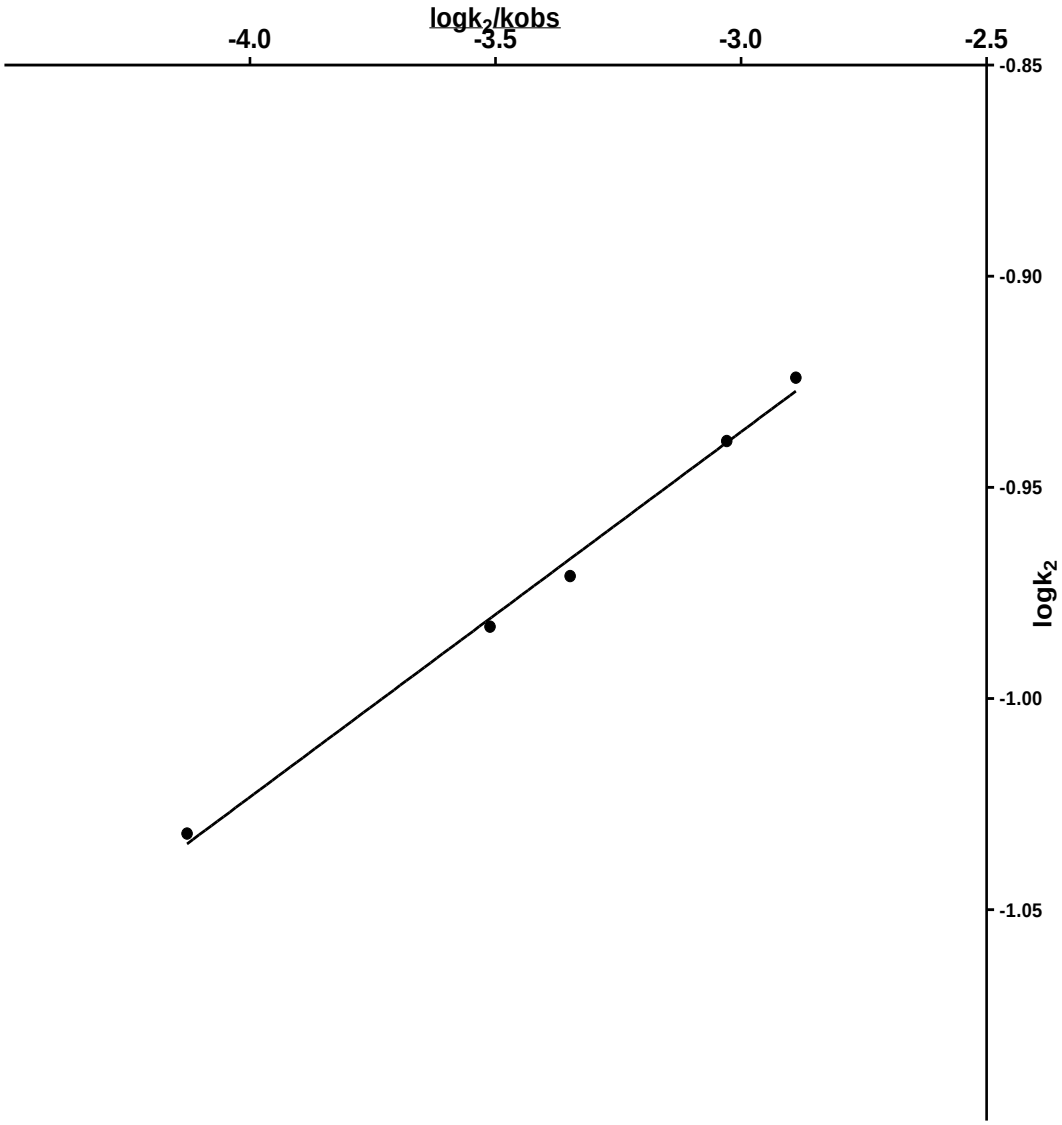


Figure 4.14: Plot of $logk_2$ versus $logk_2/k_{obs}$ for the reaction between formaldehyde and plasma albumin in water system at constant conditions [formaldehyde] = $2.7x10^{-1}$ mol dm^{-3}, [plsma albumin] = $0.51x\ 10^{-4}$ mol dm^{-3}, at varying ionic strength , $I^{½}$ = (0.1 - 0.9) mol dm^{-3} , λ_{max} = 235 nm and T= 37.0 ^{0}C in water system

Table 4.13 : The effect of ionic strength on the rate constants for the reaction of plasma albumin and formaldehyde at varying ionic strengths and constant conditions, [formaldehyde] = $0.27\ x10^{-1}$, [plsma albumin] = $0.51x\ 10^{-4}$ mol dm^{-3}, $I^{1/2}$

= (0.1 - 0.9) mol dm^{-3} , λ_{max} = 245nm,$[H^+]$=1.0 x 10^{-6} mol dm^{-3} and T= 37^0C in 15% (ethanol-water) system

$I^{½}$	k_1s^{-1}	$k_2dm^3mol^{-1}$ s^{-1}	log k_2	lnk_2/k_{obs}
0.1	0.038	0.141	-0.850	22.39
0.3	0.037	0.137	-0.863	23.32
0.5	0.035	0.129	-0.889	25.40
0.7	0.035	0.129	-0.889	25.40
0.9	0.033	0.122	-0.914	27.70

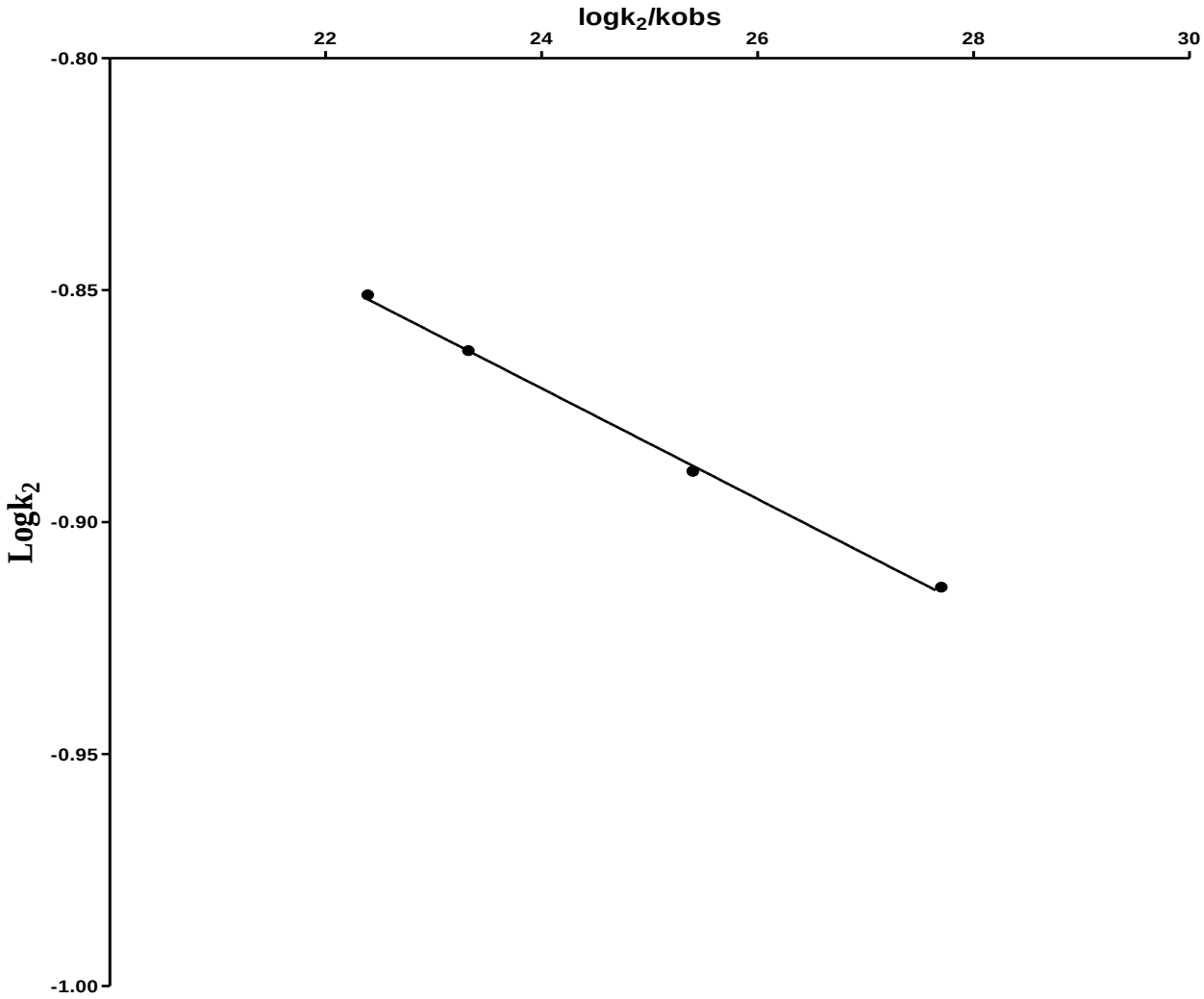


Figure 4.15: Plot of $logk_2$ versus $logk_2/k_{obs}$ showing the effect of ionic strength on the rate constants of the reaction of formaldehyde and plasma albumin in 15% (ethanol-water) system, [formaldehyde] = 0.27 x 10^{-1} mol dm^{-3} , [Plsma Albumin] = 0.51x 10^{-4} mol dm^{-3}, at varying ionic strength, $I^{1/2}$ = 0.1- 0.9 mol dm^{-3}, λ_{max} = 245 nm , pH= 7.3 and T= 37^0C in 15% (ethanol-water) system

4.6.8 Temperature dependence studies in water system

The results of the temperature studies are as presented in Tables 4.14 and 4.15 and Figures 4.1 6 and 4.1. The result in Table 4.14 show a second order rate constant but unlike in most reactions in which the rate increases with temperature, the increase was not sharp this could be due to the denaturation of the plasma albumin substrates which are responsible for the reaction, hence decrease in the concentration of the plasma albumin. The mean second order rate constant of 0.105 (± 0.00981) $dm^3 mol^{-1}s^{-1}$ at the varying temperatures (20 - 45) ^0C is less than the average second order rate constant value of 0.117 (±0.034) $dm^3 mol^{-1} s^{-1}$ at 37^0C.

Table 4.15 gives values of the activation parameters obtained from the Arrhenius and Erying plots as presented in Figures 16 and 4.17, for the temperature dependence studies. The results show a small positive Gibbs free energy and small negative values for enthalpy and entropy and activation energy respectively.

4.6.9 Temperature dependence studies in 15% and 20% (ethanol - water) systems

Tables 4.16 - 4.18 show the pseudo first and second order rate constant results for the reaction in 15% and 20% ethanol-water media and the activation parameters obtained from the plots of Arrhenius and Erying equations from the two media as presented in in fFgures (4.18 - 4.21). The rate constants were observed to decrease steadily with increase in temperatures The mean second order rate constant of 0.104 (±0.4) $dm^3 mol^{-1}s^{-1}$ at the varying temperatures, 20 - 45^0C was obtained. The results also showed a small positive Gibbs free energy of activation and activatiobn energy while negative values for enthalpy and entropy of reactions are observed in both 15% and 20% ethanol-water mixtures.

Table 4.14: Experimental results of pseudo - first order and second order rate constants at [HCHO] = 2.7 x 10^{-1} mol/dm^3, [plasma albumin] = 5.1x10^{-4} mol/dm^3, [H^+]=1.0 x10^{-6} mol/dm^3 at different temperature in water system

T(K)	$10^{-2}k_1$ s^{-1}	k_2 dm^3 mol^{-1} s^{-1}	$10^{-3}1/T$	lnk_2	$10^{-3}k_2/T$	ln (k_2/T)
293	**0.310**	**0.1148**	**3.41**	**-2.165**	**40.37**	**-3.210**
298	**0.301**	**0.1115**	**3.36**	**-2.194**	**37.20**	**-3.322**
303	**0.299**	**0.1107**	**3.29**	**-2.201**	**36.68**	**-3.306**
308	**0.295**	**0.1093**	**3.25**	**-2.214**	**35.52**	**-3.338**
310	**0.292**	**0.1081**	**3.23**	**-2.225**	**34.92.**	**-3.355**
313	**0.250**	**0.0926**	**3.19**	**-2.379**	**29.54**	**-3.522**
318	**0.243**	**0.0900**	**3.14**	**--2.408**	**28.26**	**-3.566**
306.143		**0.105 (±0.00981) dm^3 mol^{-1} s^{-1}**				

Table 4.15: Activation parameters ($\Delta G^{\#}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$) and Ea for the reaction of formaldehyde with plasma albumin in water system at different temperatures (20 - 45^{0}C)

[HCHO] mol dm^{-3}	Ea (kJ mol^{-1})	$\Delta G^{\neq}$ (kJ mol^{-1})	$\Delta H^{\neq}$ (kJ mol^{-1})	$\Delta S^{\neq}$ (J $mol^{-1}K^{-1}$)
2.7 $x10^{-1}$	-0.00807	0.0695	-0.011	--263.022

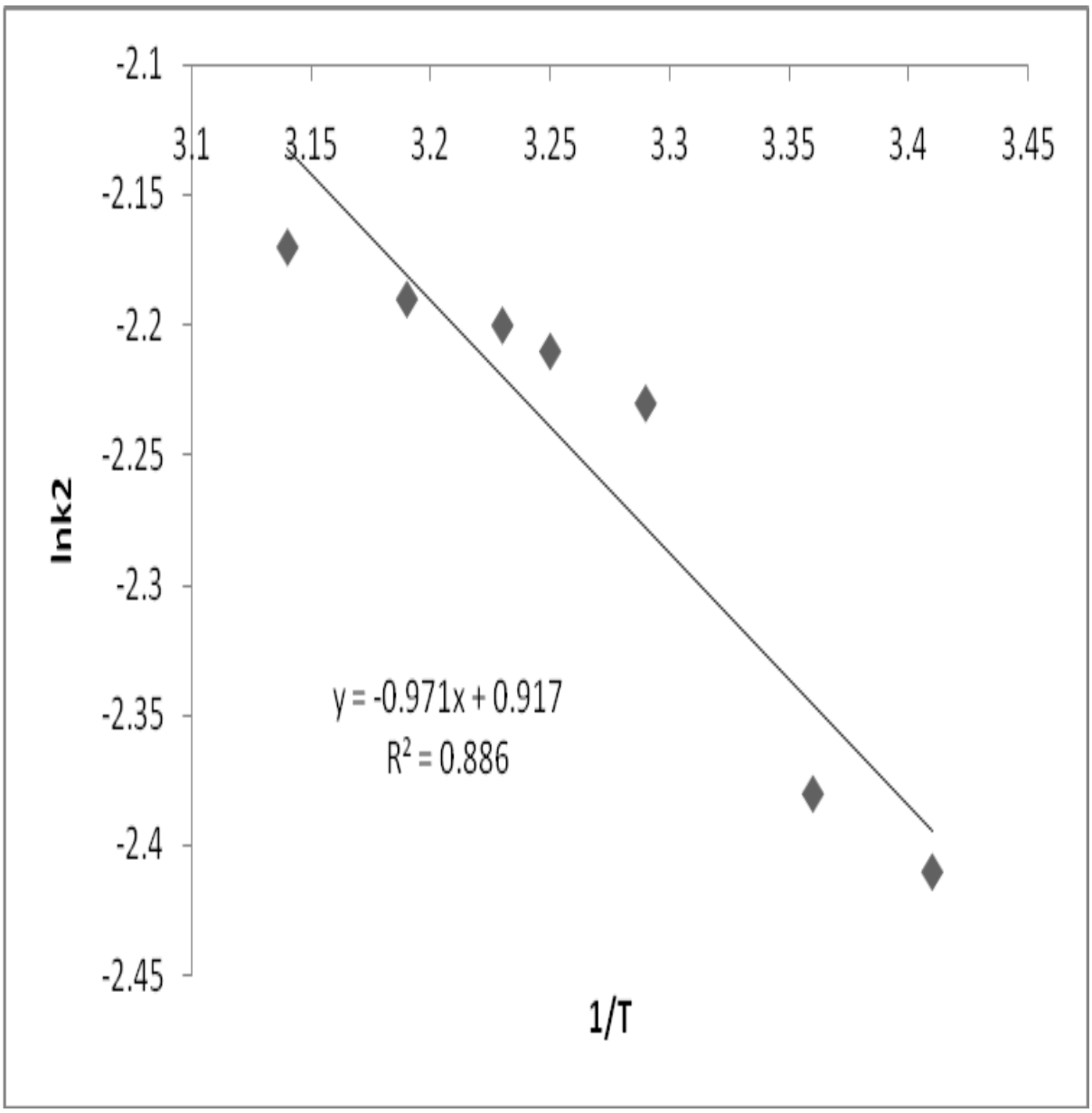


Figure 4.16: Arrhenius plot of lnk_2 versus 1/T in water system

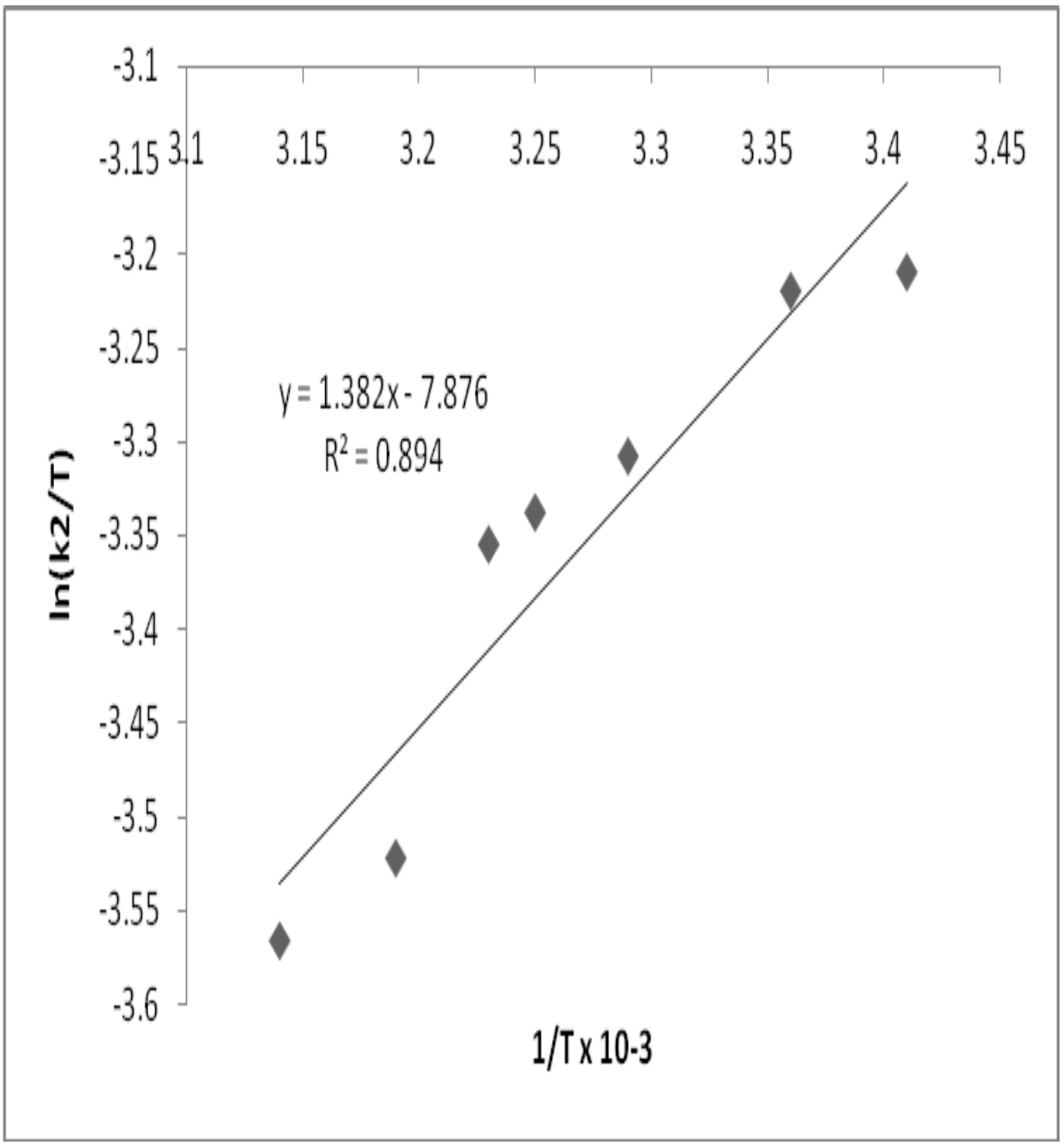


Figure 4.17 : Erying plot of ln(k_2/T) versus 10-3 x 1/T in water system

Table 4.16 Pseudo - first and second order rate constants at [HCHO] = 2.7 x 10^{-1}mol/dm^3, [plasma albumin] =5.1x10^{-4} mol/dm^3, reaction at different

temperatures in 15% (ethanol-water mixture), $I^{1/2}$ = 0.5 moldm^{-3}, [H^+]= 1.0 x 10^{-6} mol dm^{-3}

Temp(K)	$10^{-2}k_1$ s^{-1}	k_2 dm^3 $mol^{-1}s^{-1}$	10^{-3}/T	lnk_2	10^{-3} k_2/T	ln (k_2/T)
293	0.32	0.119	3.41	-2.129	40.58	-3.204
298	0.30	0.111	3.36	-2.198	37.30	-3.289
303	0.29	0.107	3.29	-2.235	35.20	-3.453
308	0.28	0.104	3.25	-2.263	33.80	-3.387
310	0.27	0.100	3.23	-2.303	32.30	-3.433
313	0.27	0. 094	3.19	-2.364	29.99	-3.507
318	0.26	0.093	3.14	-2.375	29.20	-3.534
Mean:		**0.104 (±0.4) mol^{-1}dm^3 s^{-1}**				

Table 4.17: Activation parameters ($\Delta G^{\#}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$) and Ea for the reaction of formaldehyde with plasma albumin in 15%(ethanol - water) mixture at different temperatures (20 – 45 ^{0}C)

[HCHO] mol dm^{-3}	**Ea (kJ mol^{-1})**	**$\Delta G^{\neq}$ (kJ mol^{-1})**	**$\Delta H^{\neq}$ (kJ mol^{-1})**	**$\Delta S^{\neq}$(J $mol^{-1}K^{-1}$)**
2.7 $x10^{-1}$	0.0077	0.0070	-0.00977	-258.092

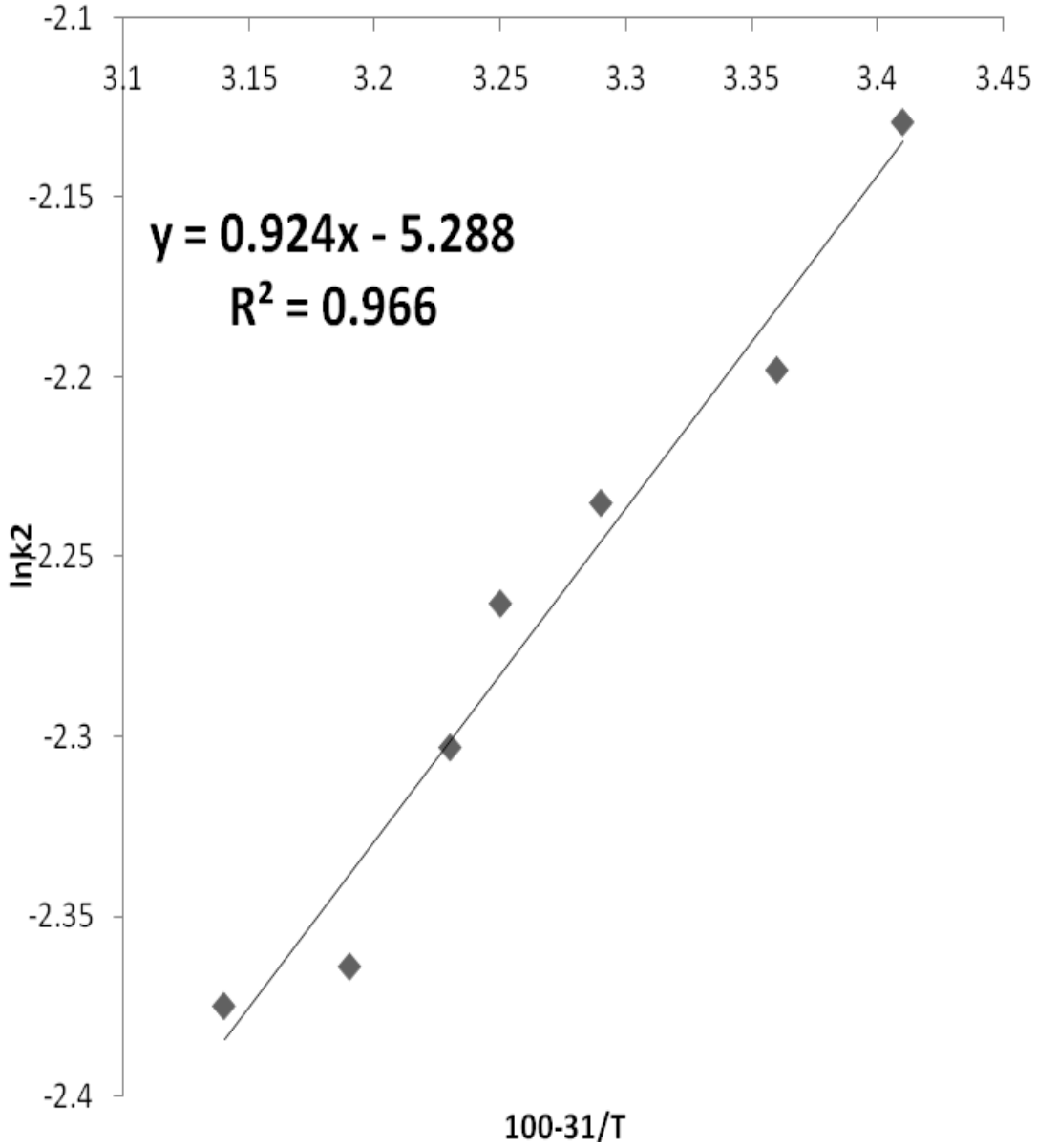


Figure 4.18: Arrhenius plot of $\ln k_2$ versus 1/T in 15 % (ethanol - water) mixture system

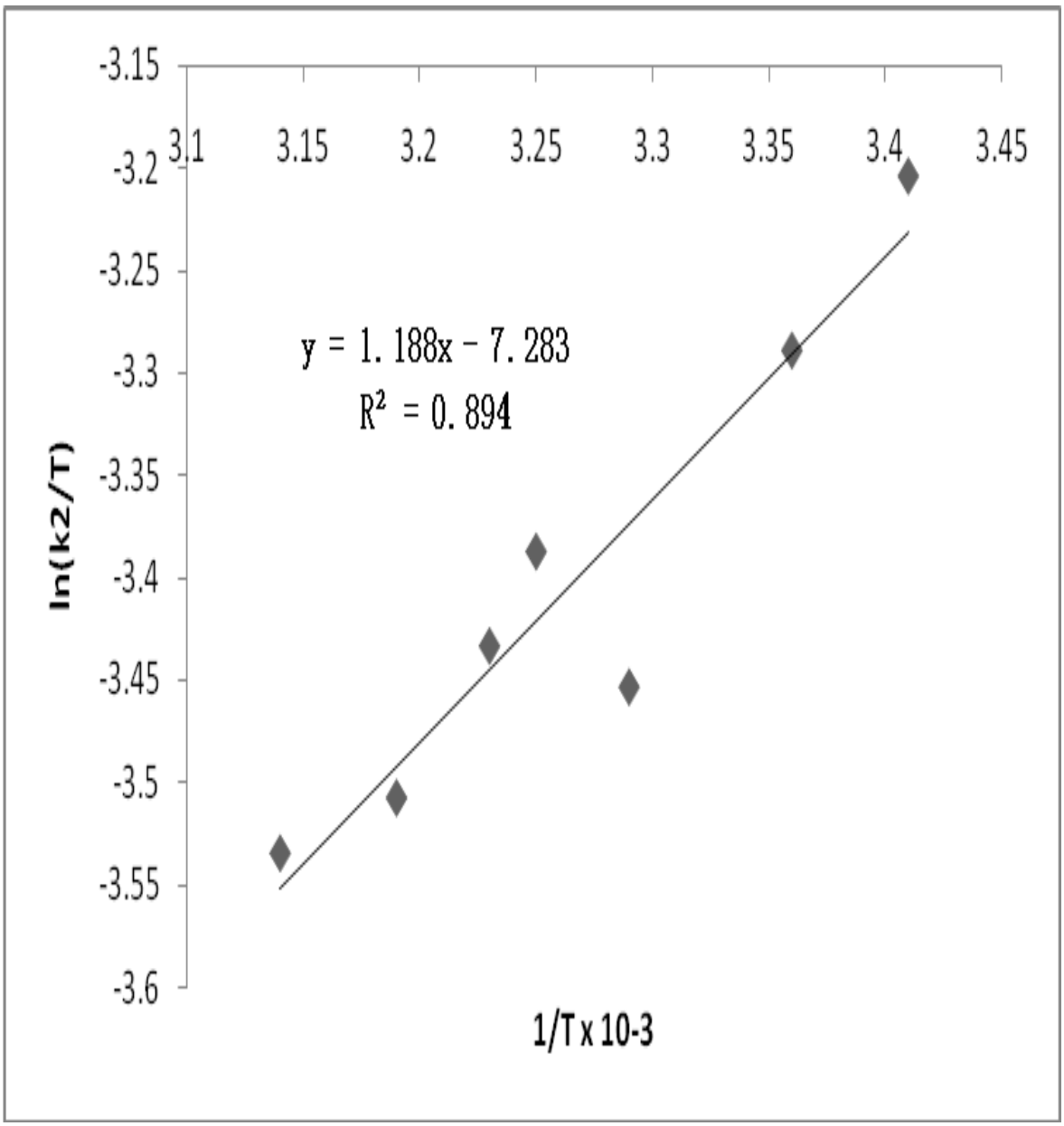


Figure 4.19: Erying plot of ln (k_2/T) versus 10^{-3} x 1/T in 15 % (ethanol - water) mixture system

Table 4.18: Experimental results of pseudo - first and second order rate constants at [formaldehyde] = 2.7 x 10^{-1} mol/dm^3 and [plasma albumin] = 0.51 x 10^{-3} mol dm^{-3} at different temperatures and 20% ethanol-water mixtures.

Temp(K)	$10^{-2}k_1$ s^{-1}	k_2 dm^3 mol^{-1} s^{-1}	$10^{-3}/T$	lnk_2	10^{-3} k_2/T	ln (k_2/T)
293	0.32	0.119	3.41	-2.129	40.58	-3.204
298	0.30	0.111	3.36	-2.198	37.30	-3.289
303	0.29	0.107	3.29	-2.235	35.20	-3.453
308	0.28	0.104	3.25	-2.263	33.80	-3.387
310	0.27	0.100	3.23	-2.303	32.30	-3.433
313	0.27	0..094	3.19	-2.364	29.99	-3.507
318	0.26	0.093	3.14	-2.375	29.20	-3.534

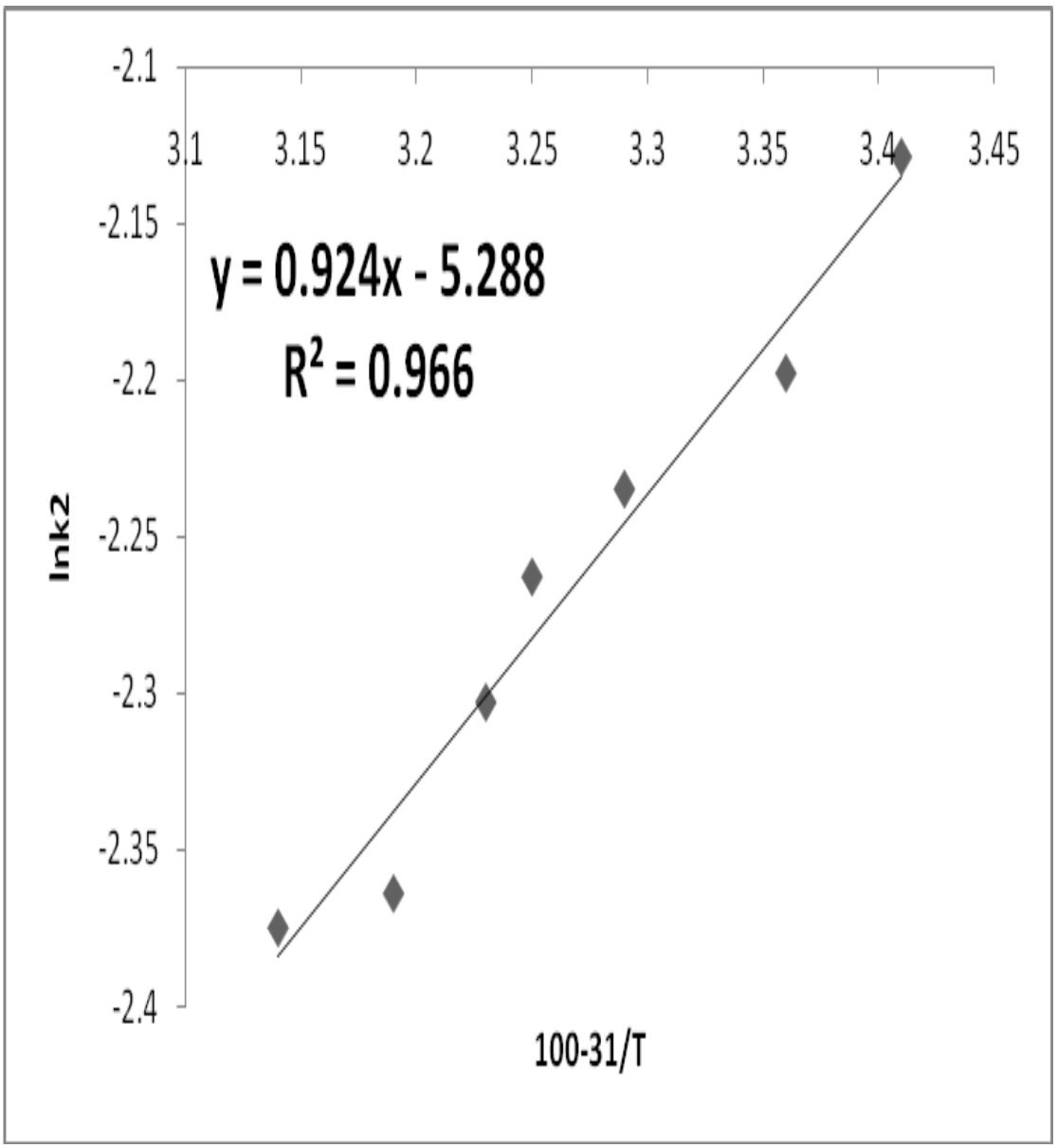


Figure 4.20: Arrhenius plot of lnk_2 versus 1/T in 20% (ethanol - water) mixture system

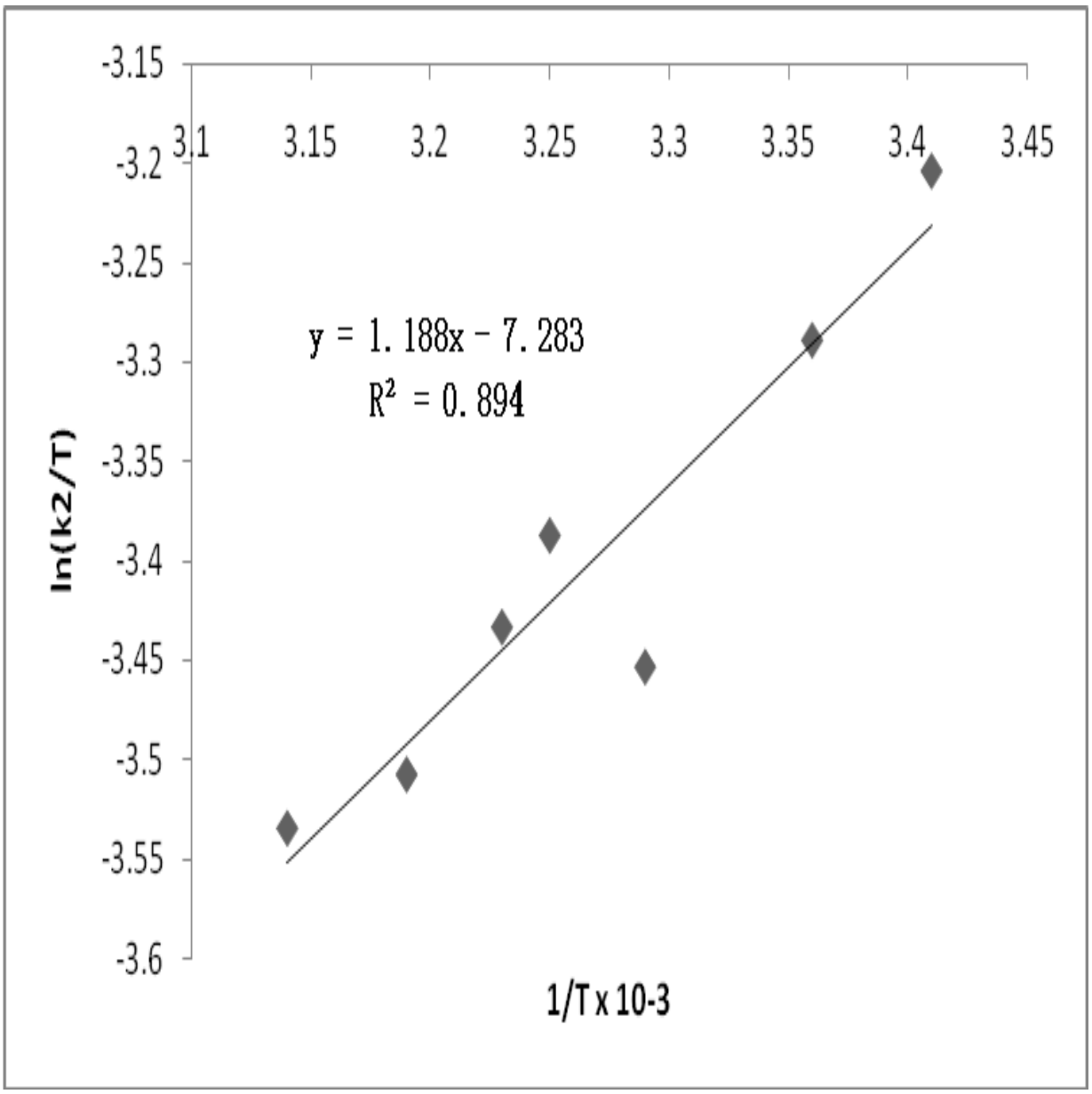


Figure 4.21 : Erying plot of ln(k_2/T) versus 10-3 x 1/T in 20 % (ethanol - water) mixture system

Table 4.19: Activation parameters ($\Delta G^{\#}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$) and Ea for the reaction of formaldehyde with plasma albumin in 20% (ethanol -water) mixture at different temperatures (20 – 45 ^{0}C)

[HCHO] mol dm^{-3}	Ea (kJ mol^{-1})	$\Delta G^{\neq}$ (kJ mol^{-1})	$\Delta H^{\neq}$ (kJ mol^{-1})	$\Delta S^{\neq}$(J mol^{-1}K^{-1})
2.7 x10^{-1}	0.00768	0.070	-0.00977	-258.092

4. 6.10 pH dependence studies in water system

Table 4.20 and Figure 4.22 show the effect of change in hydrogen ions concentration on the rate constant values for the kinetic interaction between plasma albumin and formaldehyde in water.. The rate constants of the reactions in water medium increased with increase in pH. This shows that the rate constant was found to be acid dependent in the range investigated. A Plot of $\ln k_2$ versus $[H^+]$ gave a linear graph of slope 0.109 with a correlation coefficient of 0.906. The acid – dependent rate constant for the reaction in water solution can be represented by equation (Li *et al.,* 2007)

$$k_2 = a + b\,[H^+] \quad \text{---------- 4.3}$$

where 'a' and ' b' represents intercept and slope respectively.

4.6.11 Effect of added anions and cations on the reaction rate

The effect of added anions (CO_3^{2-} *and* NO_3^-) and cations (Fe^{3+}, Ca^{2+} and Zn^{2+}) in the range 0.05 - 1.0 mol dm^{-3} were found to enhanced the reaction in the water solution system studied.. The results are presented in Table 4.21 and 4.22. The results of the plots of rate constants versus the concentrations of the anions and cations, CO_3^{2-} *and* NO_3^- and Fe^{3+}, Ca^{2+} and Zn^{2+} are as presented in Figures .4.23 - 4.27 respectively. All thesse plots show linear relation ship with the added anions and cations.as their concentrations increased.

Table 4.20: Effect of change in hydrogen ions concentration on the rate constants for the reaction of plasma albumin and formaldehyde at Varying $[H^+]$ and constant conditions in water medium, [formaldehyde] = 2.7 x10^{-1} moldm^{-3}, [plasma

albumin] = 0.51x 10^{-4} mol dm^{-3}, $I^{1/2}$= 0.5 moldm^{-3}, $[H^+]$ = 5.0 x 10^{-6} - 1.99 x 10^{-9} mol dm^{-3}, λ_{max} = 245nm and T= 37.0 ^{0}C

pH	$10^{-9}[H^+]$	k_{obs} s^{-1}	k_2 dm^3 mol^{-1} s^{-1}	lnk_{obs}
5.3	0.005	0.024	0.089	-3.729
6.4	0.039	0.028	0.104	-3.576
6.8	0.016	0.028	0.104	-3.576
7.3	0.501	0.031	0.115	-3.474
8.3	5.012	0.031	0.115	-3.474
8.7	1.995	0.036	0.133	-3.324

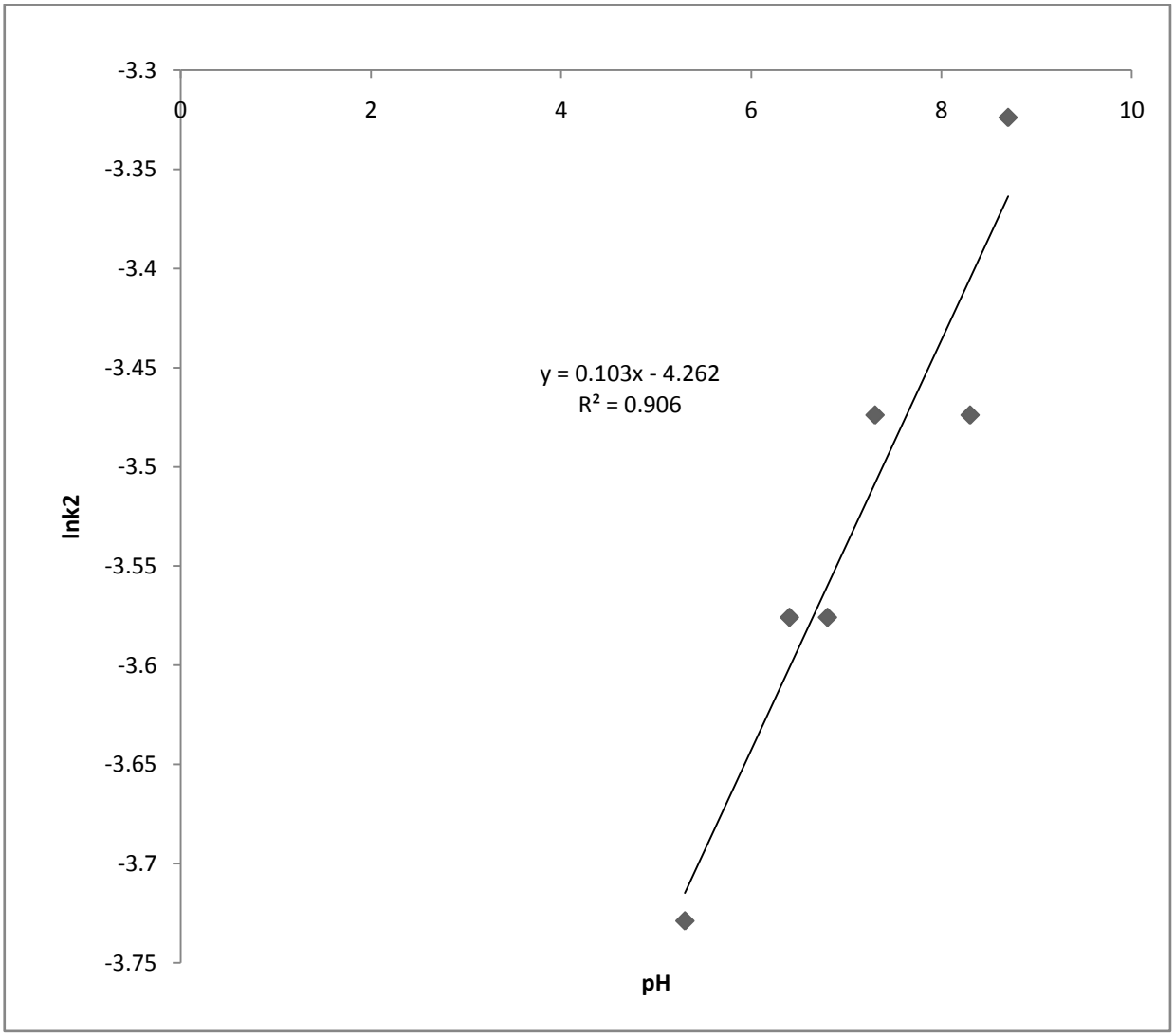


Figure 4.22: Plot of log k_2 versus $-\log_{10}[H^+]$ showing the variation of pH on the rate of reaction of formaldehyde and plasma albumin reaction in water system

Table 4.21: Rate data for the effect of added anions (CO_3^{2-} and NO_3^-) on the rate constant of plasma albumin and formaldehyde at [formaldehyde] = 2.7 x 10^{-1}

moldm^{-3}, [plasma albumin] = 0.51x 10^{-4} moldm^{-3}, λ_{max}= 245nm, [H^{+}]= 5.0x 10^{-8} moldm^{-3}, and T= 37^{0}C in Water

Ion	10^{4}[ion], mol dm^{-3}	k_1 s^{-1}	k_2 mol^{-1}dm^{3} s^{-1}
CO_3^{2-}	0.05	0.0685	0.254
	0.1	0.0726	0.269
	0.5	0.0776	0.287
	1.0	0.0812	0.301
NO_3^{-}	0.05	0.0713	0.264
	0.1	0.0723	0.270
	0.5	0.0822	0.304
	1.0	0.0842	0.312

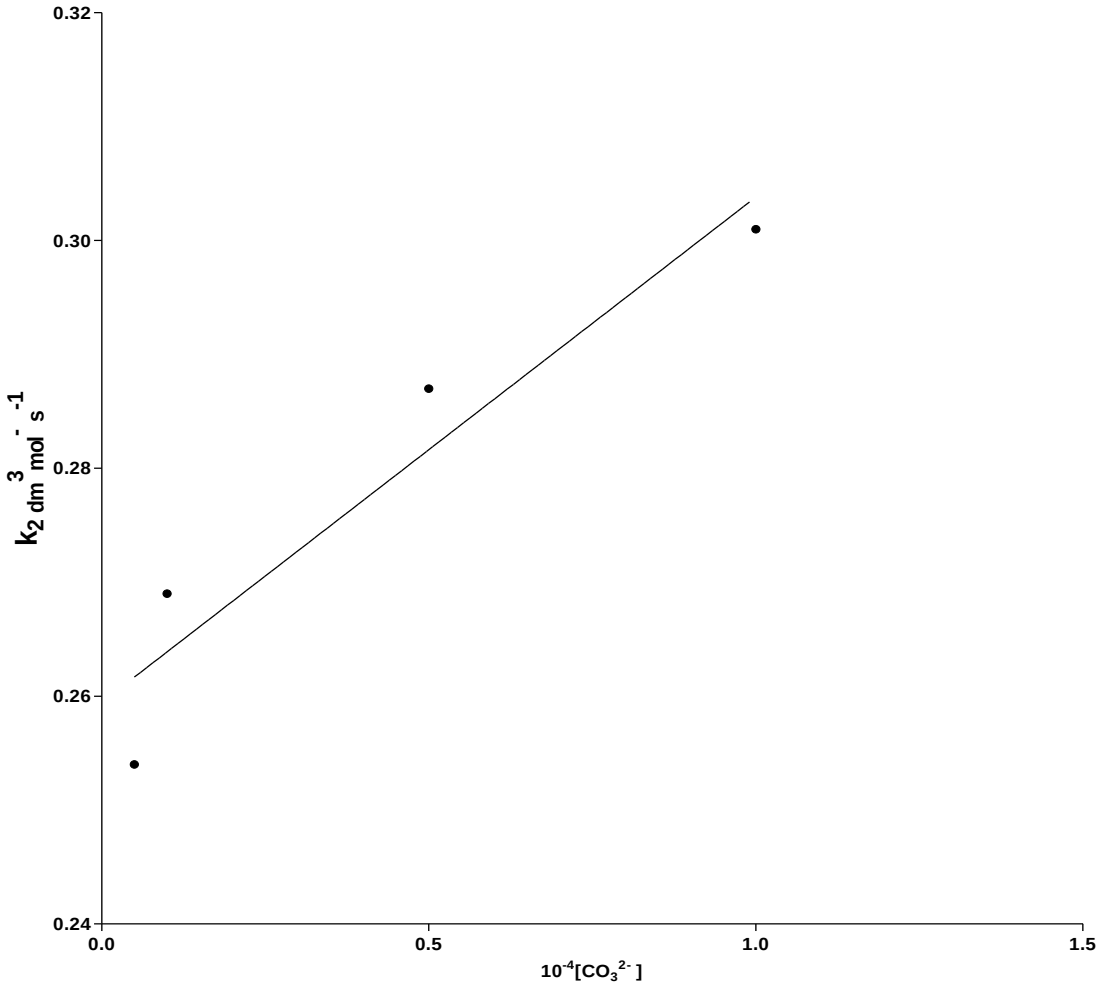


Figure 4.23: Plot of k_2 versus $[CO_3^{2-}]$ for the reaction of formaldehyde and plasma albumin in water system , [formaldehyde] = $0.27x10^{-1}$ mol dm^{-3}, [Plasma Albumin] = $0.51x\ 10^{-4}$ mol dm^{-3}, $I^{1/2}$ = 0.5 mol dm^{-3}, λ_{max} = 245 nm , pH = 7.3 and T= 37^0C

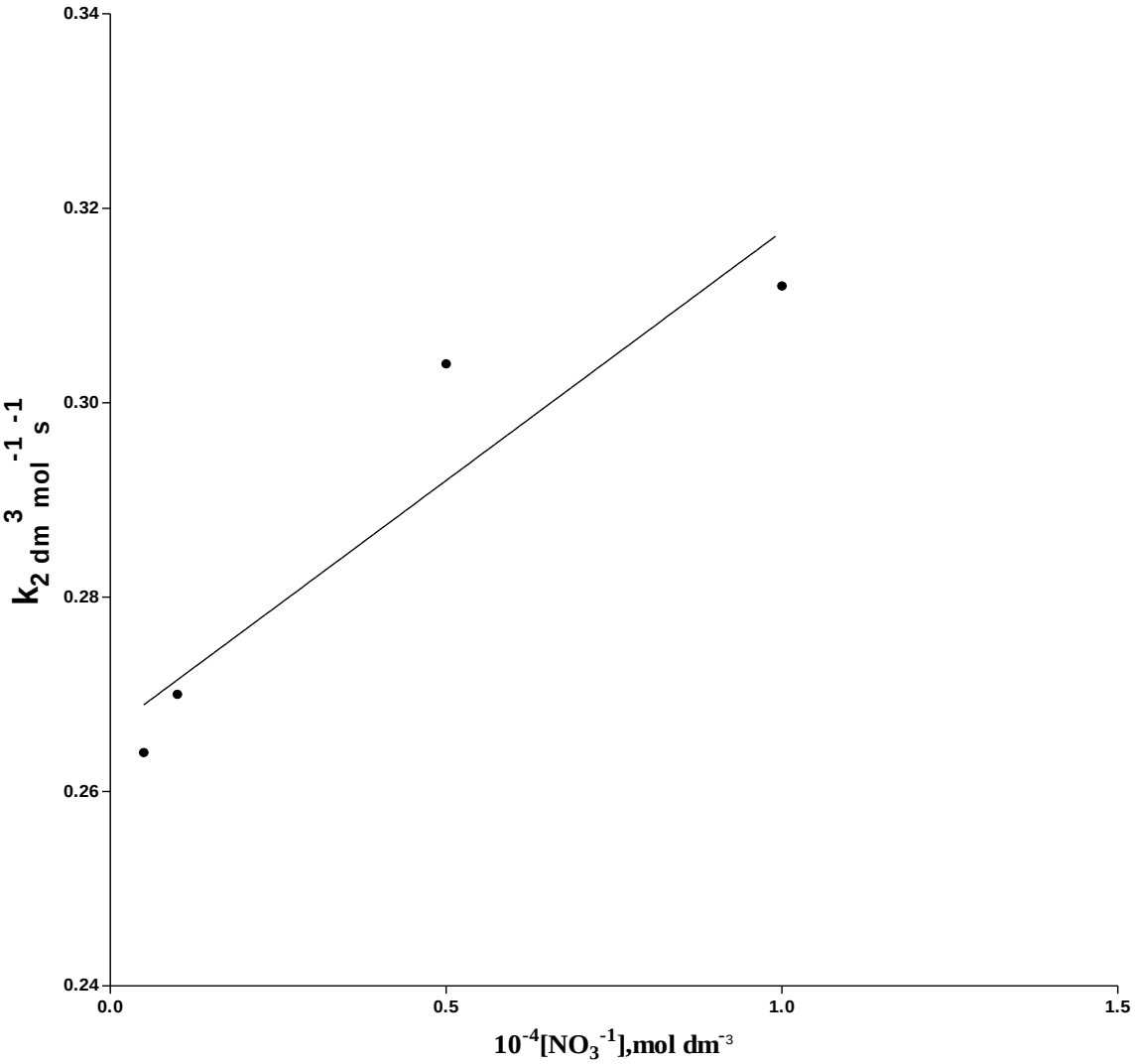


Figure 4.24: Plot of k_2 versus $[NO_3^-]$ for the reaction of formaldehyde and plasma albumin in water system, [formaldehyde] = 0.27 x 10^{-1} mol dm^{-3}, [plasma albumin] = 0.51x 10^{-4} mol dm^{-3}, $I^{1/2}$ = 0.5, λ_{max} = 245 nm , pH= 7.3 and T= 37^0C.

Table 4.22 : Rate data for the effect of added cations (Fe^{3+}, Ca^{2+} and Zn^{2+}) on the rate constant of plasma albumin and formaldehyde at [formaldehyde]= 2.7 x

10^{-1} , [plasma albumin] = 0.51x 10^{-4}, λ_{max} = 245nm , $[H^+]$ = 5.0x 10^{-8} and T= 37^0C in water

Ion	10^{-4}[ion], mol dm^{-3}	k_1 s^{-1}	k_2 $mol^{-1}dm^3 s^{-1}$
Fe^{3+}	0.05	0.0162	0.060
	0.1	0.0186	0.069
	0.5	0.0208	0.077
	1.0	0.0235	0.087
Ca^{2+}	0.05	0.0251	0.093
	0.1	0.0265	0.098
	0.5	0.0281	0.104
	1.0	0.0302	0.112
Zn^{2+}	0.05	**0.0294**	0.109
	0.1	**0.0311**	0.115
	0.5	0.0327	0.121
	1.0	0.0335	0.124

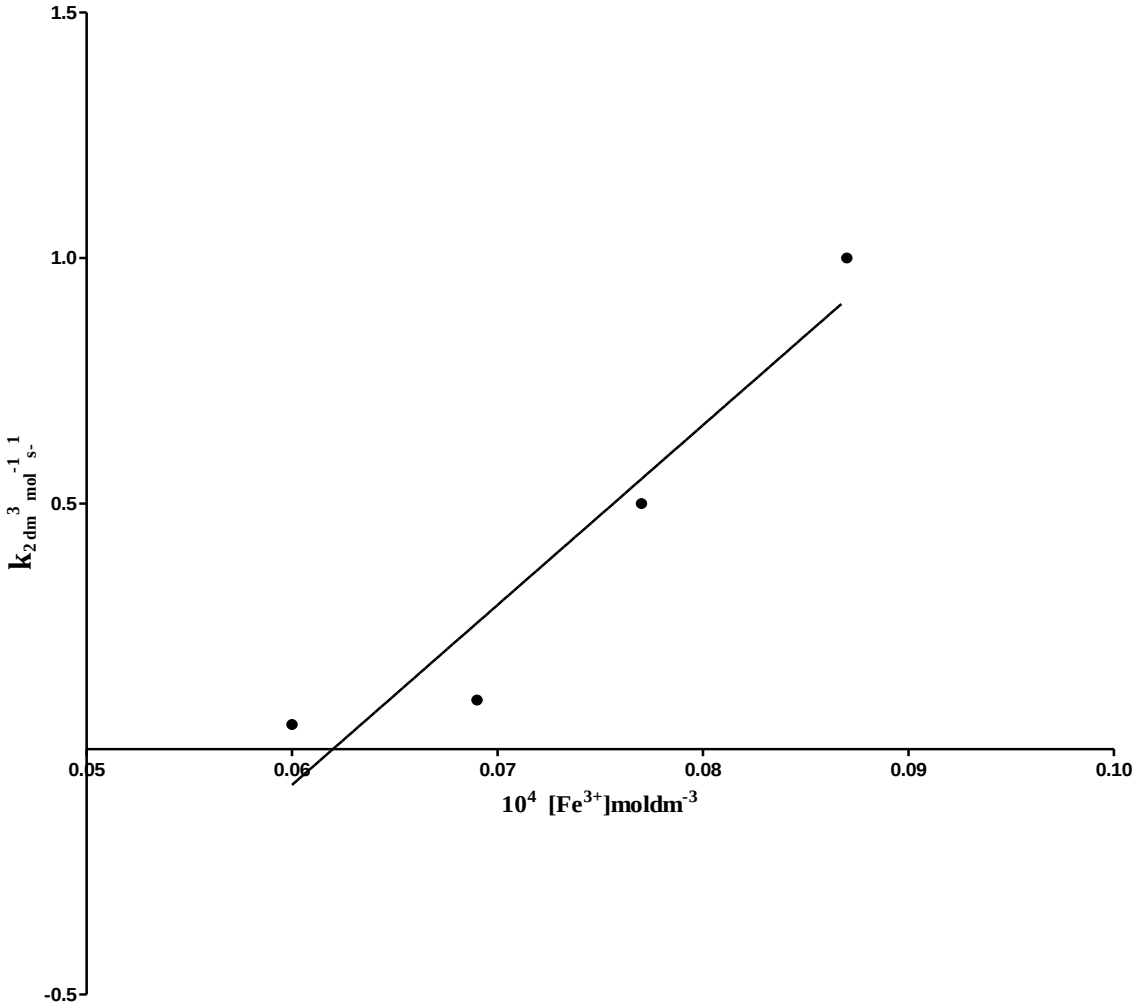


Figure 4.25: Plot of k_2 versus [Fe^{3+}] for the reaction of formaldehyde and plasma albumin in water system, [Formaldehyde]= 0.27 x10^{-1}mol dm^{-3}, [plasma albumin] = 0.51x 10^{-4} mol dm^{-3}, $I^{1/2}$ = 0.5 mol dm^{-3}, λ_{max} = 245nm , pH= 7.3 and T= 37^{0}C.

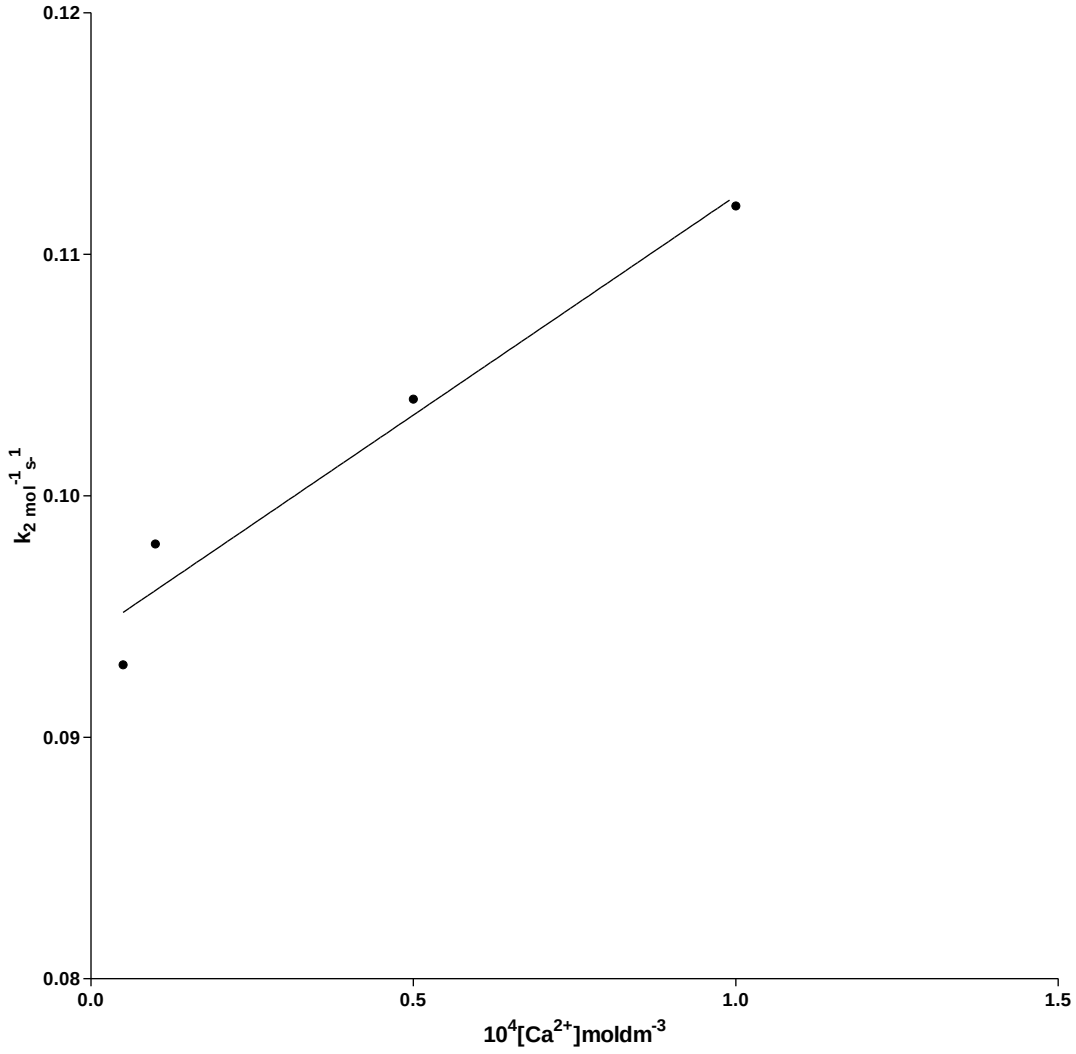


Figure 4.26: Plot of k_2 versus $[Ca^{2+}]$ for the reaction of formaldehyde and plasma albumin in water solution, [formaldehyde]= 0.27 x10^{-1} mol dm^{-3}, [plasma albumin] = 0.51x 10^{-4} mol dm^{-3} , $I^{1/2}$ = 0.5 mol dm^{-3}, λ_{max} = 245nm , pH= 7.3 and T= 37^0C.

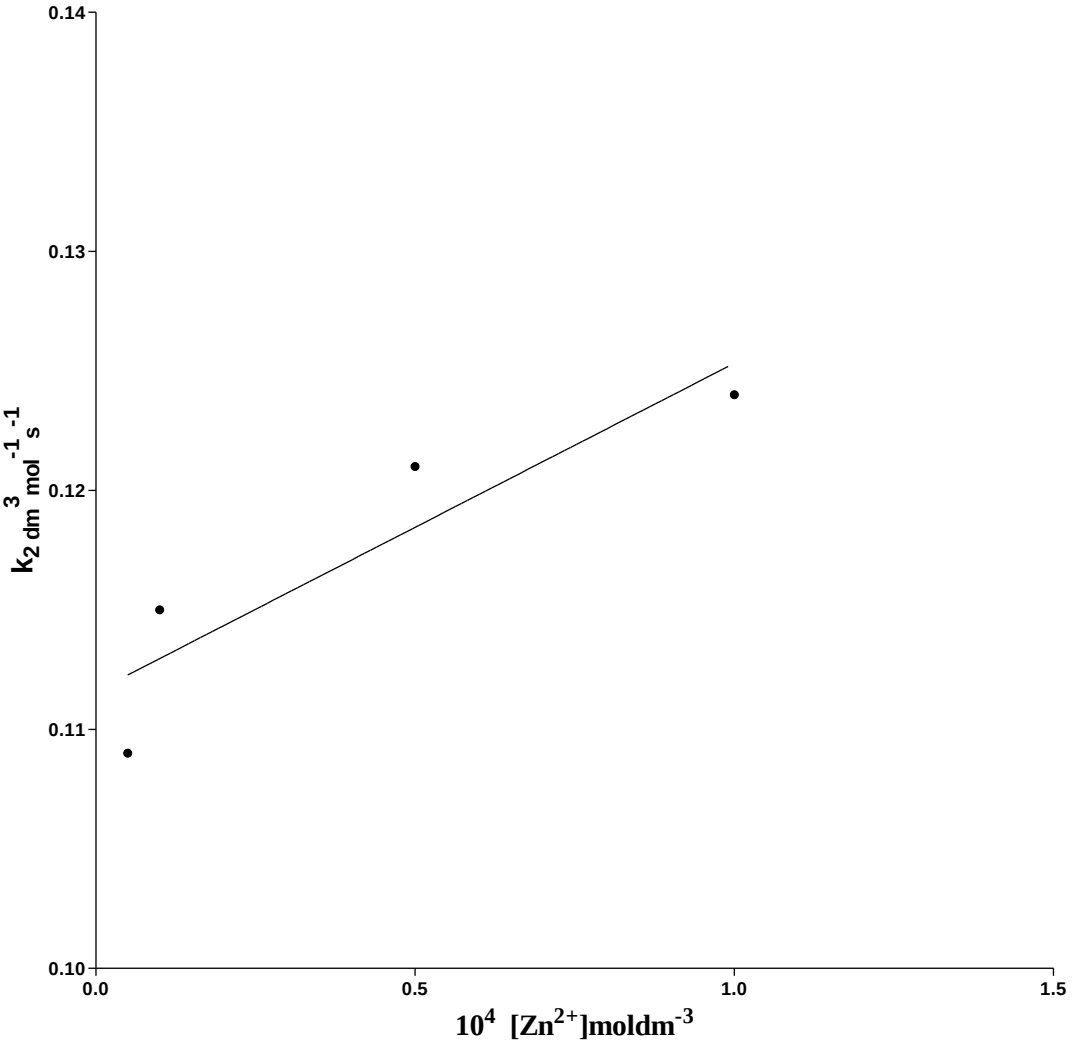


Figure 4.27: Plot of k_2 versus [Zn^{2+}] for the reaction of formaldehyde and plasma albumin in water system [formaldehyde] = 0.27 x10^{-1} mol dm^{-3} [plasma albumin] = 0.51x 10^{-4} mol dm^{-3} , $I^{1/2}$ = 0.5 mol dm^{-3}, λ_{max} = 245nm , pH= 7.3 and T= 37^0C

4.6.12: Effect of changes in dielectric constants of the reaction medium on the reaction rate

Tables 4.23 and Figures 4.28 – 4.31 present the data for rate constant of plasma albumin and formaldehyde reaction at various compositions of ethanol in water. The values of rate constants were found to decrease with increase in the concentration of ethanol. This deviation from the Br**o**nsted theory is in accordance to the literature (Uddin *et al.*, 2006). The activation energies and the activation parameters were found to be affected by solvent medium. The activation energy and the activation parameters (Gibbs free energy, enthalpy and entropy changes of the reaction at the activated complex) decreased with decrease in dielectric constant of the medium (that is with ncrease in the proportion of the ethanol component in the solvent mixtures). Table 4.24 and figure 4.31 is results of a two tailed correlation analysis for k_2 values versus ethanol - mixtures at 95% level of assurance. The k_2 values linearly decreased with decreased in dielectric constant with a p value 0.0033.

Table 4.23: The dependence of the reaction rate constants with % ethanol-water mitures carried out at constant reactants concentration [HCHO] = $0.27x10^{-1}$mol dm^{-3}, [$PABNH_2$] = $5.1x\ 10^{-4}$ mol dm^{-3}, $I^{1/2}$ = 0.5 mol dm^{-3}, pH = 7.0 ± 3 and T= 37

%EtOH	%H_2O	[EtOH]	[H_2O]	k_1	k_2	logk_2	log H_2O	χ_{EtOH}	χ_{H2O}	$D10^{-2}$	1/D	pKa
0.0	100	0.0	55.5				1.74	0.00	1.000	78.5	1.27	0.00
1.0	99.0	0.17	55.0	0.03	0.115	-0.94	1.74	0.003	0.997	78.0	1.28	12.3
5.0	95.0	0.86	52.8	0.02	0.101	-0.99	1.72	0.016	0.984	75.8	1.32	13.3
10.0	90.0	1.72	50.0	0.03	0.100	-1.00	1.69	0.033	0.967	73.1	1.37	13.6
15.0	85.0	2.58	47.22	0.03	0.085	-1.07	1.67	0.052	0.948	70.4	1.42	13.8
20.0	80.0	3.44	44.44	0.03	0.081	-1.10	1.65	0.072	0.928	67.6	1.48	13.9
25.0	75.0	4.30	41.67	0.03	0.080	-1.10	1.62	0.094	0.906	64.9	1.54	14.1

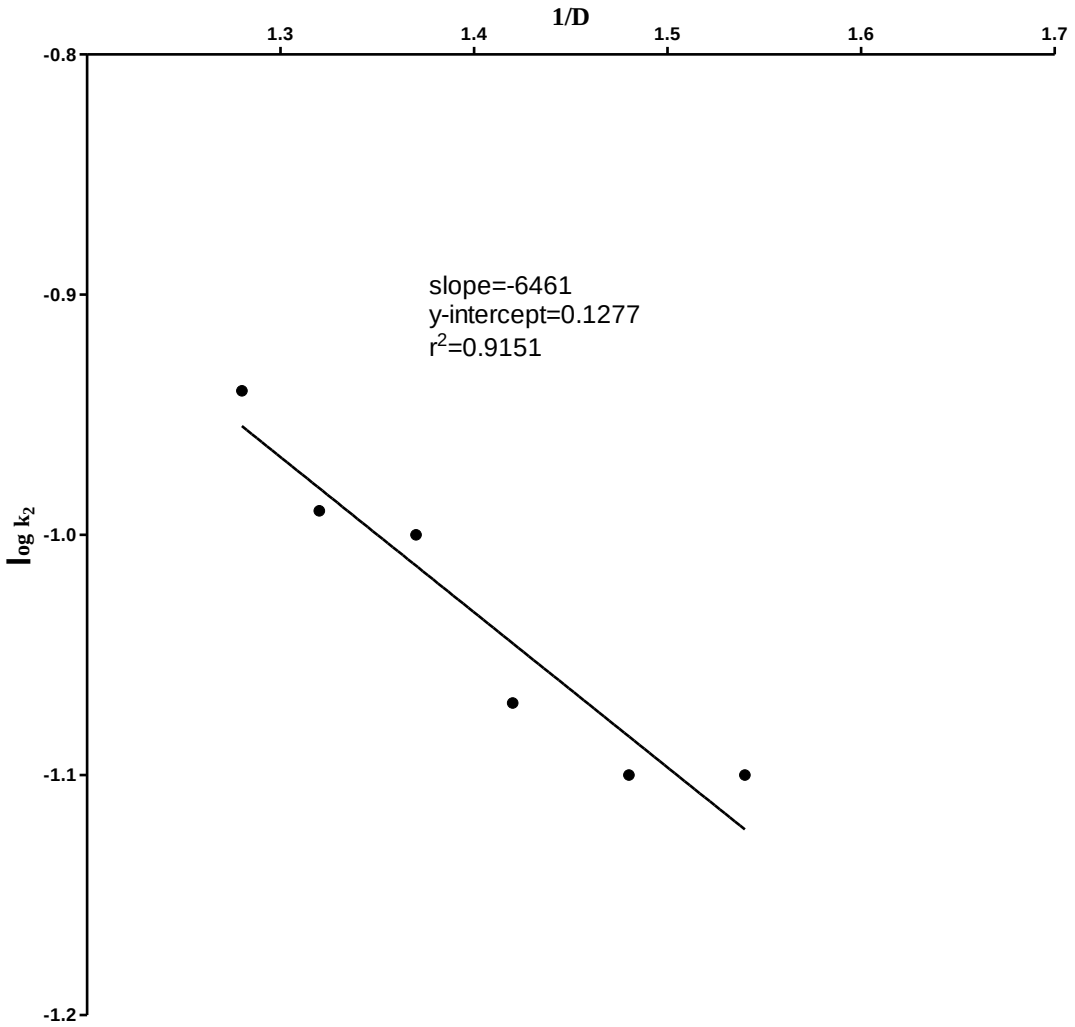


Figure 4.28: Plot logk_2 versus 1/D showing the effect of change in dielectric constants on the rate constant of the reaction of plasma albumin with formaldehyde in %(ethanol-water) mitures carried out at constant reactants concentration [HCHO] = 0.27x10^{-1} mol dm^{-3}, [$PABNH_2$] = 5.1x 10^{-4} mol dm^{-3}, $I^{1/2}$ = 0.5 mol dm^{-3}, [H^+]= 5.0 x 10^{-8}mol dm^{-3}, and T= 37^0C

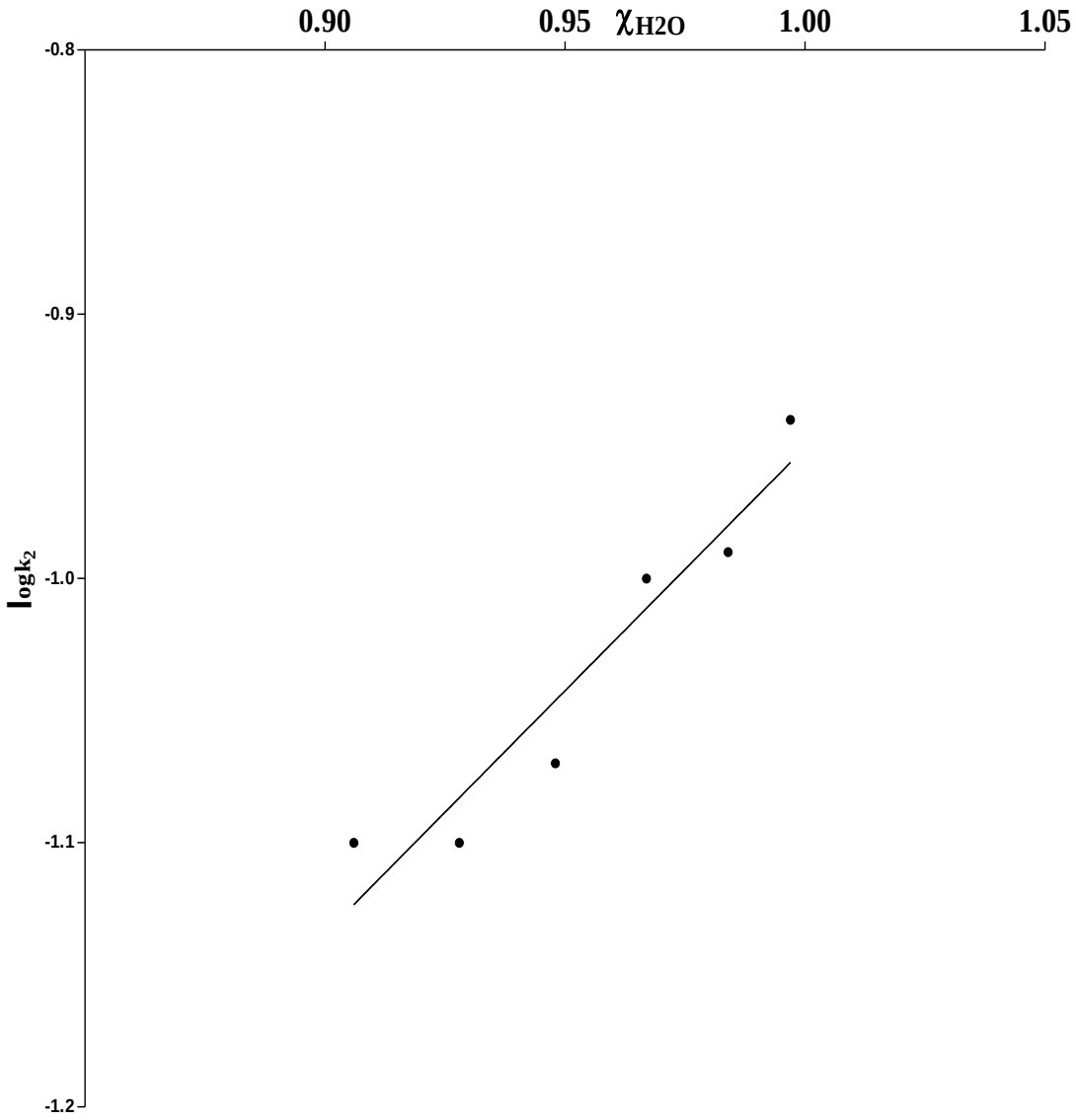


Figure 4.29: Plot of $logk_2$ versus mole fractions of water (χH_2O)

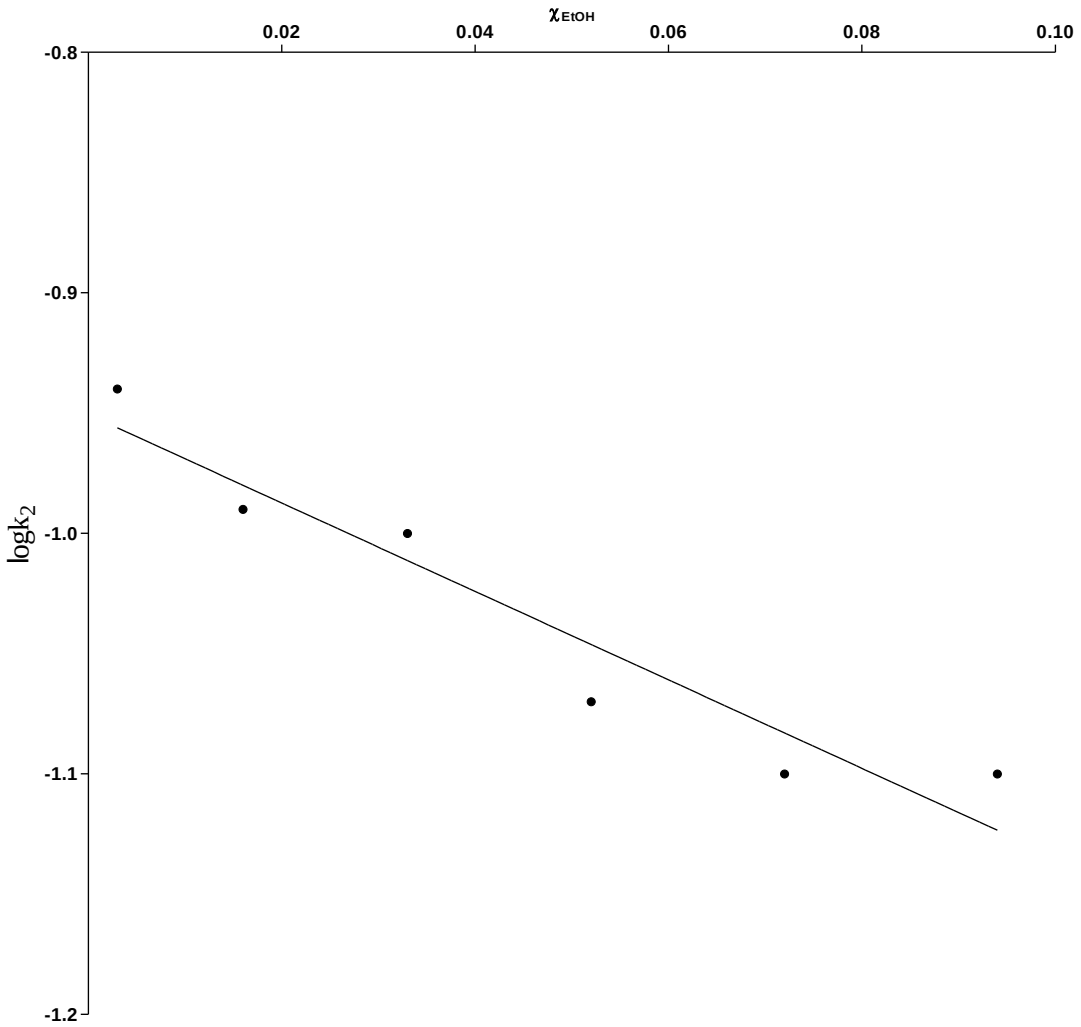


Figure 4.30: Plot of $\log k_2$ versus mole fractions of ethanol - water mixtures

Table 4.24: results of two tailed correlation analysis for k_2 values versus ethanol mixtures at 95% level of assurance

Best-fit values	
Slope	**-0.001460 ± 0.0002329**
Y-intercept when X= 0.0	**0.1122 ± 0.003527**
X-intercept when Y=0.0	**76.80**
1/slope	**-684.7**
95% Confidence Intervals	
Slope	**-0.002107 to -0.0008139**
Y-intercept when X=0.0	**0.1024 to 0.1220**
X-intercept when Y=0.0	**56.88 to 128.0**
Goodness of Fit	
r^2	**0.9077**
Sy.x	**0.004735**
Is slope significantly non-zero?	
F	**39.32**
DFn, DFd	**1.000, 4.000**
P value	**0.0033**
Deviation from zero?	**Significant**
Data	
Number of X values	**6**
Maximum number of Y replicates	**1**
Total number of values	**6**
Number of missing values	**0**

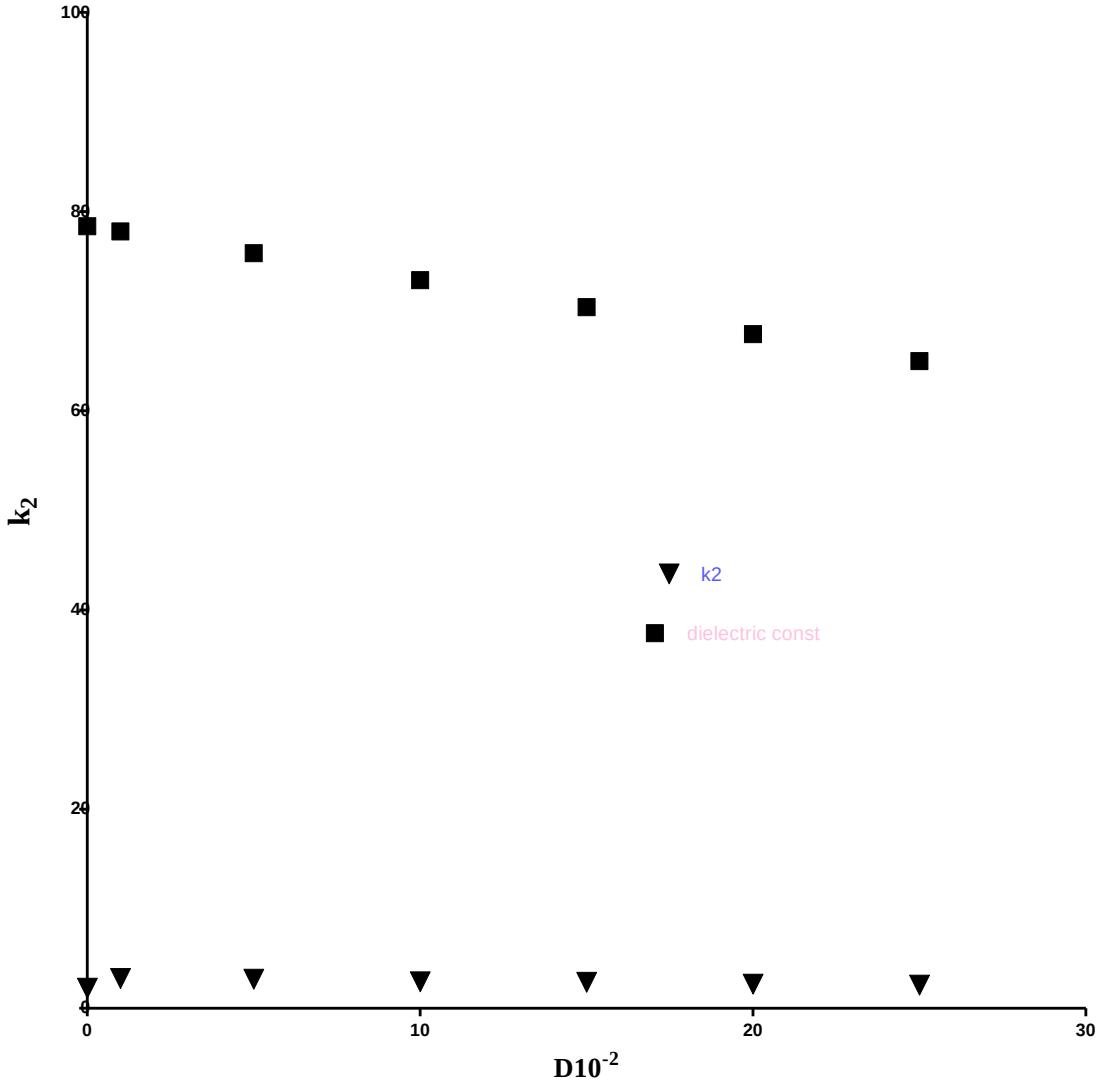


Figure 4.31: Correlational analysis chart of k_2 values versus dielectric constants at different ethanol-water mixtures

4.6.13 Results of the kinetic study of reactions of plasma albumin with formaldehyde in ethanol - water mixtures and water solution using Bronsted-type plot

Tables 4.25 - 4.32 and figure 4.37 present the experimental data of the effect of pH on the absorbance of plasma albumin and formaldehyde-plasma albumin adduct at varying pH and constant conditions in ethanol – water mixtures and at temperature of 37.0 ^{0}C. The result of the absorbance differences were used to calculate the molar absorptivity and reciprocal absorptivity values as well as the hydrogen ion concentrations [H^+]. The molar absorptivity values increased with increase in the pH values. The pKa values of plasma albumin used in the study was obtained from a plot of its absorption vesus pH. As shown in Figure 4.37. The pKa values obtained fall within the pH region 6.0 - 8.0 Similarly, Tables 4.27 and 4.28 show the literature and the experimental pka data of some amino acids and plasma albumin with formaldehyde adduct and they were found to be in agreement.The results of the Bronsted plots of $logk_2$ versus pka for plasma albumin-formaldehyde adduct in both ethanol-water mixtures and water solution are as presented in figures 4.38 and 4.39. The results show the β_{nuc} slope values of β = 0.071 and 0..0195 respectively.

Table 4.25: Experimental data of the effect of pH on the absorbance of plasma albumin and formaldehyde - plasma albumin adduct at varying pH and constant conditions in water, [formaldehyde] = 0.27 x10^{-1}mol dm^{-3}, [plasma albumin] = 0.51x 10^{-4} mol dm^{-3}, $I^{1/2}$ = 0.5 mol dm^{-3}, pH= 5.3 - 8.0 , λ_{max} = 235 nm and T= 37.0 ^{0}C

pH	Absorbance		ΔAbs_{235}	$\Delta\varepsilon_{235}$ $M^{-1}cm^{-1}$	$1/\Delta\varepsilon_{235}$	$[H^+]$	%Eth -H_2O	D	k_2
	$PABNH_2$	HCHO $PABNH_2$							
5.3	1.445	1.480	0.035	2541.76	$3.93x10^{-4}$	$5.01x10^{-6}$	1	78.0	2.40
6.4	1.450	1.497	0.047	3413.22	$2.93\ x10^{-4}$	$3.98x10^{-7}$	5	75.5	2.50
6.7	1.463	1.520	0.057	4139.43	$2.42x10^{-4}$	$1.99x10^{-7}$	10	73.1	2.56
6.9	1.471	1.543	0.072	5228.76	$1.91\ x10^{-4}$	$1.26x10^{-7}$	15	70.4	2.59
7.3	1.475	1.556	0.081	5882.35	$1.70\ x10^{-4}$	$5.01x10^{-8}$	20	67.6	2.63
8.0	1.500	1.596	0.096	6971.68	$1.43\ x10^{-4}$	$1.0\ x10^{-8}$	25	64.9	2.67

Effect of pH on k_2 for the reaction of formaldehyde with plasma albumin using m 2.7×10^{-1} mol/dm^3 HCHO at 37 ^{0}C and varying Ethanol-water mixtures.

Table 4.26: Experimental data of the effect of pH on the absorbance of plasma albumin and formaldehyde-plasma albumin adduct at varying pH and constant conditions in ethanol - water mixtures , T = 37.0 ^{0}C , [formaldehyde] = 0.27×10^{-1} mol dm^{-3}, [plasma albumin] = $0.51x\ 10^{-3}$ mol dm^{-3} $I^{1/2}$ = 0.5 $moldm^{-3}$, pH= (5.3 - 8.0) , λ_{max} = 235 nm

pH	Absorbance	ΔAbs_{235}	$\Delta\varepsilon_{235}$	$1/\Delta\varepsilon_{235}$	$[H^+]$	k_2	$\log k_2$

	$PABNH_2$	HCHO-$PABNH_2$						
5.3	1.470	1.490	0.020	1452.43	6.89×10^{-4}	5.01×10^{-6}	0.69	-0.16
6.4	1.472	1.493	0.021	1525.05	6.56×10^{-4}	3.98×10^{-7}	0.82	-0.09
6.7	1.475	1.497	0.022	1597.68	6.26×10^{-4}	1.99×10^{-7}	0.86	-0.07
6.9	1.480	1.503	0.023	5670.30	1.76×10^{-4}	1.26×10^{-7}	0.86	-0.07
7.3	1.484	1.508	0.024	5882.35	1.70×10^{-4}	5.01×10^{-8}	0.98	-0.01
8.0	1.488	1.514	0.026	6971.68	1.43×10^{-4}	1.0×10^{-8}	1.11	0.005

Effect of pH on k_2 for the reaction of formaldehyde with plasma albumin using 2.7×10^{-1} mol/dm^3 HCHO at 37^0C 37^0C in water

Table 4.27: pKa values of some amino acids and dielectric constant values of ethanol-water mixtures (D =78.0 - 64.9) at 37^0C, pH= 5.3 -8.4 and [$I^{1/2}$] = 0.5 [NaCl] from literature

%Eth-H_2O	[Eth-H_2O] mol dm^{-3}	pKa $M^{-1}cm^{-1}$	[H_2O)] mol dm^{-3}	Eth-H_2O Dielectric Constant
1	0.17	8.61	55.00	78.0

5	0.86	8.50	52.8	75.5
10	1.72	8.31	50.00	73.1
15	2.58	8.21	47.22	70.4
20	3.44	8.13	44.44	67.6
25	4.30	8.05	41.67	64.9

pKa values from the data of Tong (2003).

Table 4.28: Experimental pKa and second order rate constant values of plasma albumin and plasma albumin-formaldehyde adduct from ethanol - water mixtures at T= 37^0C, pH= 5.3 - 8.0 and $I^{1/2}$ = 0.5mol dm^{-3}

%Ethanol-Water	Eth-H_2O moldm^{-3}	pKa $M^{-1}cm^{-1}$		Dielectric constant of water	k_2 mol dm^{-3}	log k_2
		$PABNH_2$	HCHO-$PABNH_2$			
1	0.17	5.5	1.0	55.00	2.40	0.380

5	0.86	6.5	2.1	52.8	2.50	0.398
10	1.72	7.0	3.1	50.0	2.56	0.408
15	2.58	7.3	4.2	47.22	2.59	0.413
20	3.44	7.5	5.8	44.44	2.63	0.420
25	4.30	8.0	6.0	41.67	2.67	0.427

Mean pKa = 6.97

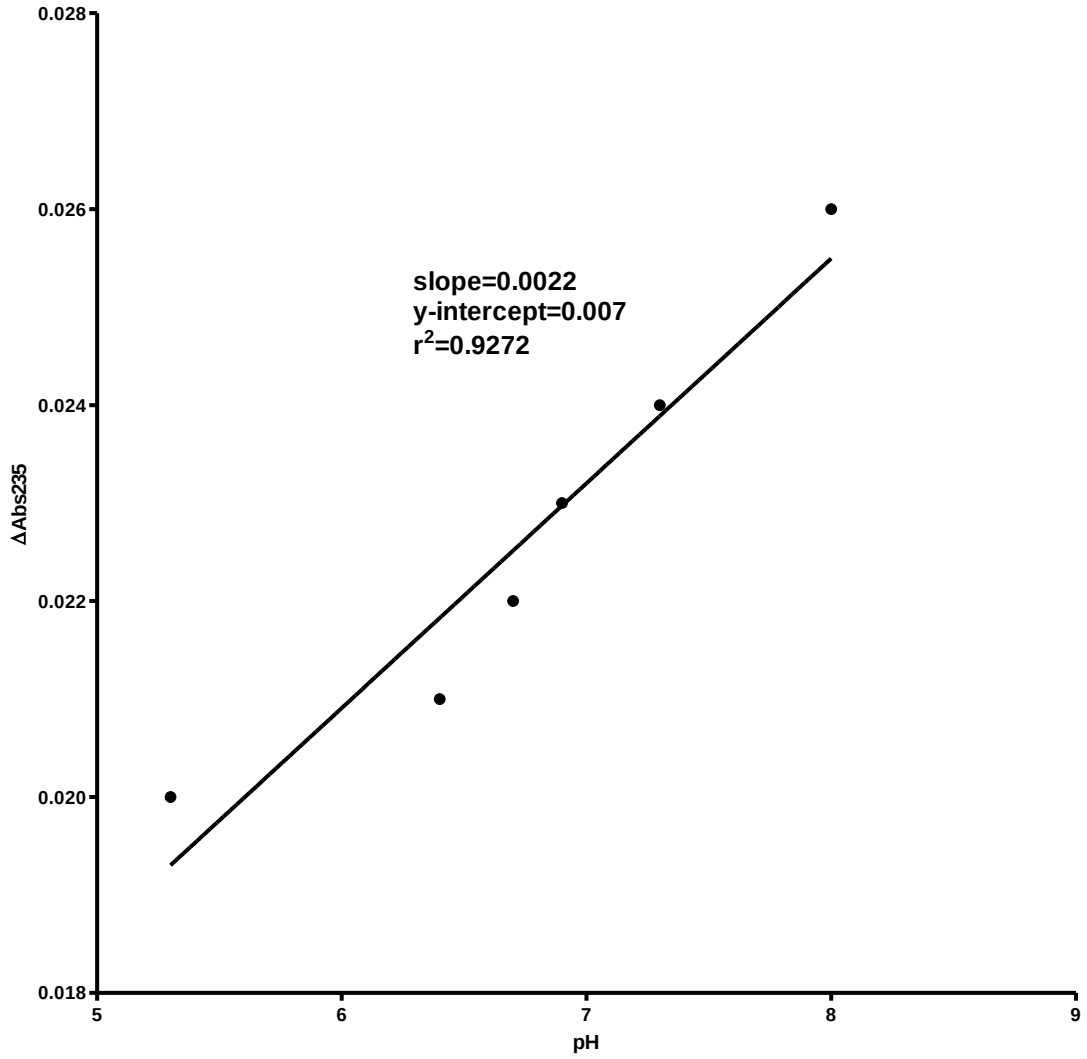


Figure 4.32: pKa values of plasma albumin from a plot of molar absorptivity versus pH for the reaction of plasma albumin with formaldehyde in varying ethanol-water mixtures at T= 37 ^{0}C and pH= 5.3 – 8.0

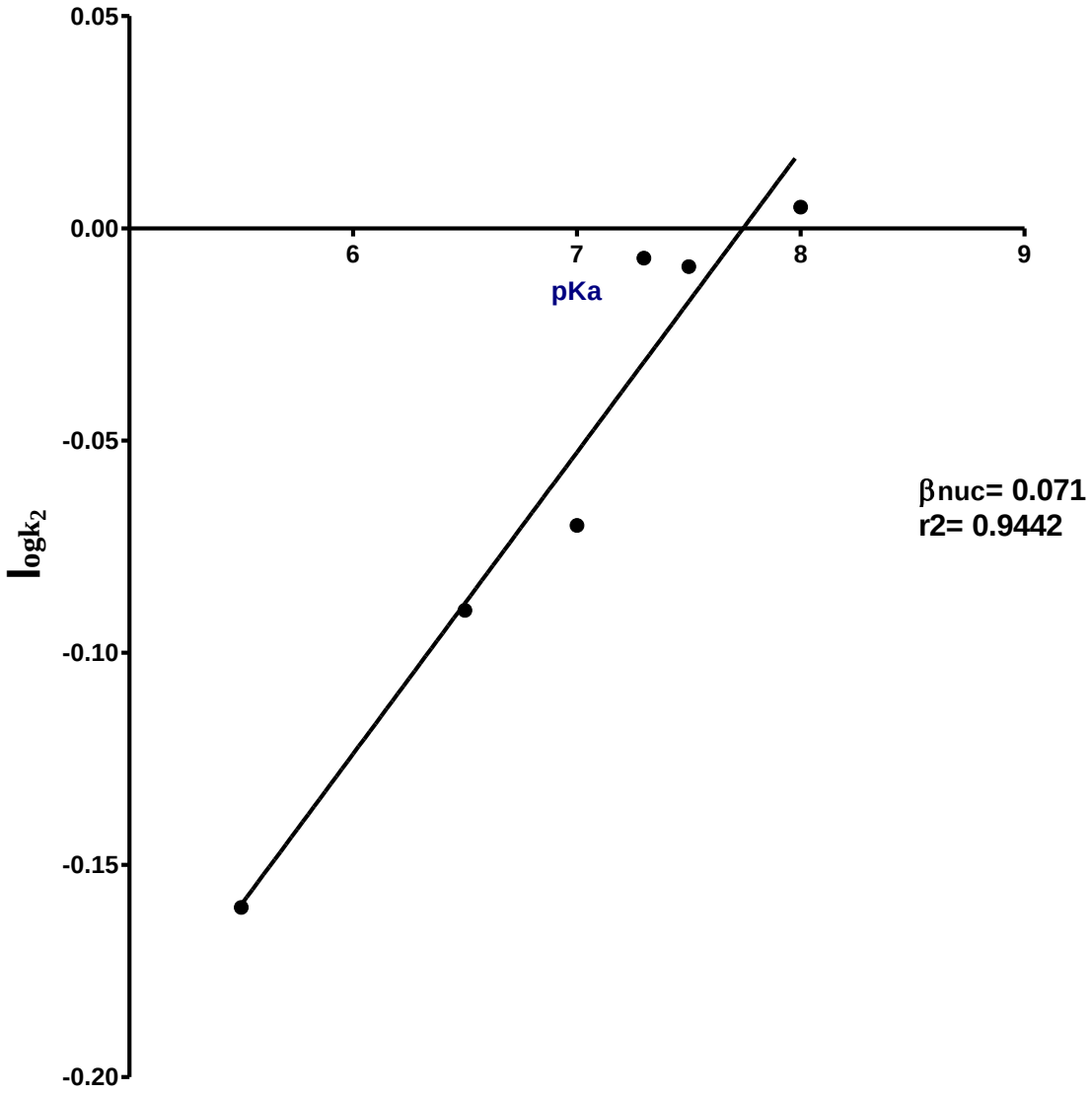


Figure 4.33: Bronsted- type plot of $logk_2$ versus pKa of plasma albumin in the reaction between plasma albumin and formaldehyde in water system at T= 37 ^{0}C and pH = 5.3 – 8.0

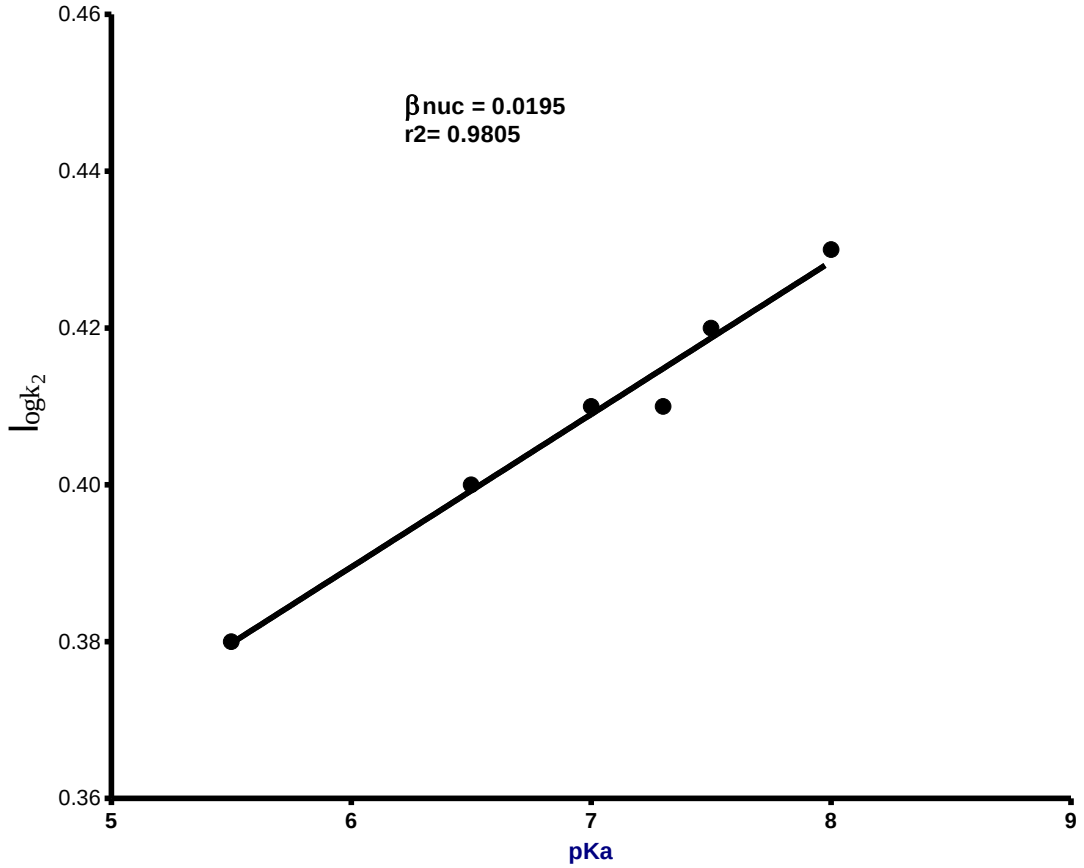


Figure 4.34: Bronsted-type plot of log k_2 versus pKa of plasma albumin-formaldehyde adduct in the reaction between plasma albumin and formaldehyde in varying ethanol - water mixtures (1.0 -25%) at T= 37^{0}C and pH = 5.3 – 8.0

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 Analysis of Formaldehyde Levels in Human Urine, Milk and Food- Related Samples

The formaldehyde levels from human milk, urine, fish and milk products as determined by Hantzsch spectrophotometric method using Acetylacetone and acetoacetanilide reagents and subjected to correlational analysis reveal that both methods gave significant formaldehyde values in all the samples that were analysed, that is: in human milk, urine, fish, milk products and the cosmetics samples using the two reagents at $p< 0.0001$) and coefficient of variation value of 0.9925. This shows that the acetoacetanilide reagent has no superiority over acetylacetone reagent and the results are similar.The results obtained also were found to be insignificant compared to maximum acceptable value of 0.2% for skin cosmetic products and the reported toxic value of $0.08g/100cm^3$ HCHO in the body for humans.

5.2 Analysis of Formaldehyde Levels in Cosmetics and Non – Food Materials

The formaldehyde values obtained from the cosmetic materials and non-food materials were also studied using acetoacetanilide and acetylacetone reagents (both ß-ketones). These were also determined at room temperature based on the Hantzsch reaction. The detection reactions involved the cyclization between acetoacetinalide and formaldehyde in the presence of ammonia that was derived from ammonium citrate at 365 – 368 nm at room temperature to result to a 1, 4 dihydropyridine dicarboxylate while that between acetylacetone and formaldehyde in the presence of ammonia derived from ammonium citrate that was measured at 400 - 415nm Li

_et al.,_ (2007) formed a 3,5-diacetyl 1,4- dihydrolutidine. Botth reagents show appreciable but low levels of formaldehyde in g/Kg in the cosmetics and non-food samples relative to the WHO toxic limits for foods and non foods samples in the two reagents. The results also show that the both reagents used gave comparable values of formaldehyde in the samples analysed as shown in correlational analysesm in Table 4.3 and Figure 4.2.

According to IARC (1995) 1he most widely used methods for the determination of the concentration of formaldehyde in air and other environmental niches are based on spectrophotometry, with which sensitivities of 0.01–0.03 mg/m3 can be achieved. Other methods include colorimetry, fluorimetry, high-performance liquid chromatography (HPLC), polarography, gas chromatography (GC), infrared detection and gas detector tubes. Most methods require the formation of a derivative for separation and detection. Methods for the analysis of formaldehyde in biological matrices (e.g. blood andurine) are rere but also have been reviewed (ATSDR, 1999), and new methods continue to be reported (Luo _et al.,_ 2001; Kato _et al.,_ 2001). (Heck _et al.,_ 1982, 1985 reported that Formaldehyde has been measured in blood by gas chromatography-mass spectrometry (GC–MS) after derivatization to pentafluorophenylhydrazone Formic acid or formate iwhich is produced from formaldehyde and can be measured in blood and urine (Baumann and Angerer, 1979). However, biological monitoring of exposure to formaldehyde is reported not to be a common practice. Based on the foregoing information and from the results of the formaldehyde levels obtained from the food related and non food samples in this study by these methods, we have demonstrated that the method used in this study are reliable and could be used in the routine analyses of variable samples including biological samples

5.3 Characterization oF Isolated Plasma Albumin

The results of characterization oF the solated plasma albumin and prepared aduct by percentage recovery, optical rotation, solubility in water, denaturation test and %protein content are as presented in chapter four. The isolated plasma albumin sample showed a protein content of 63.67%, with a very poor percentage recovery of 1.36% (2.4g). Also the isolated albumin was observed to be slightly soluble in water and ethanol hence was slightly denatured with a specific rotation $[\alpha]_{598}^{32}$ value of 8.0 at pH 7.4. In spite of these properties the quality of plasma albumin was found to be active and pure in its native state (Michael, 1968). Also the method allowed for isolation and recovery of native state plasma albumin from simple and available reagents and equipment without resulting to the expensive and time consuming chromatographic techniques.

5.4 Ultra Violet Spectra, Melting Point, Antimicrobial Susceptibility and Infrared Spectroscopy Analyses of 1,4 - Dihydropyridine Adduct

The results of melting point, antimicrobial susceptibility and infrared spectroscopy analyses for the prepared 1,4-dihydropyridine adduct was based on the method of Hantzsch reaction (Li *et al.,* (2007) is as follows. The melting point analysis shows that the adduct had a melting point range of 179.8 - 180.4^{0}C. This indicated that the adduct is libile Schiff base and a protein with similar characteristics with those found in the literature which were referred to gylcated products (Povey et al., 2007). The result of anti microbial activity analysis shows that the 1,4-dihydropyridine adduct has some antimicrobial effect ton some micro-organisms such *as Bacillus subtilis* zone of

inhibition 12.0 mm, *Proteum mirabilis* zone of inhibition, 16.0 mm and *Canida albicans* zone of inhibition, (14.0 mm. It was also observed that the adduct had no effect on some microorganisms such as *Escherichia coli* and *Staphylococcus aureus* which are typical fungi or algae. This inhibitory property or antimicrobial property was reported to be typical of lectin action mechanisms, and it was observed that the 1.4- dihydropyridine adduct had the specialized protein properties of a glycop protein (Peumans and VanDamme, 1995) as shown in Figure 4.4.

The IR analysis showed the functional groups present in the adduct. The bands were assigned based on the principle of Hsu, (1997) as follows: The band at 2900.76cm^{-1} was assigned to aldehydic C-H stretch and the one at 3845.5 cm^{-1} was found to be (O-H) stretch for water of hydration.The sharp 2356.87 band was found to be due to methylene bridge CH_2 stretch in the adduct while the bands at 1647.2, 1517.4 cm^{-1} and 1032.63 cm^{-1} were assigned to N-H amines bend and C-N amines stretch respectively, Figure, 4.5
This point in the direction that the adduct is a protein or glycoprotein a moety comprising of both protei and carbohydrate ends or reactive siites.

5.5 Kinetic Studies

5.5.1 Wavelengths of maximum absorption (λmax) of formaldehyde, plasma albumin and formaldehyde - plasma albumin adduct

Both Formaldehyde and Plasma albumin were found to absorbed maximally very closely within the same uv region at a λ_{max} of 210 nm. Plasma albumin however was found to

also absorb more widely within the range of 198 – 250 nm and at other wavelengths (350 and 400 nm) respectively as given in Figures 4.6 and 4.7.

In view of this the kinetic measurements were carried out at λ_{max} 235 nm a wave length far removed from formaldehyde and their adduct to investigate the disappearance of plasma albumin in the reaction of plasma albumin with formaldehyde under pseudo-first order conditions.

5. 5. 2 Stoichiometry

The results of the stoichiometric analysis showed that one mole of formaldehyde is consumed by two moles of plasma albumin. A value of 2 was observed as presented in Figure 4.10 confirming that using both methods, the mole ratio of the plasma albumin to formaldehyde was 2: 1 and the all reactions in the current study obeyed pseudo-first -order kinetics. On the basis of this result the over all equation for the reaction can be represented as follows:

$$H_2C(OH)_2 + 2PABNH_2 \rightarrow \ PABN(HCH_2\ (OH)_2 - - - -4.1$$

The stoichiometric studies showed that one mole of formaldehyde react with 2 mole of plasma albumin. A stoichiometry of 1:1 has also been reported for permanganate and formaldehyde. Tkinetic study showed second order with respect to both formaldehyde and plasma Albumin.

5.5.3 Determination of order and rate constant values of the reactantin in water and ethanol-water mixtures

All the reactions were found to obeyed pseudo-first-order kinetics. Pseudo-first order rate constants (k_{obs}) were calculated from the equation,

$$\ln (A_t - A_\infty) = -k_{obs}dt + C.$$

Six different formaldehyde concentrations were used to determine the second-order rate constants (k_2) from the slope of their linear plots or from the relation:

$$k_2 = k_{obs} / [\text{HCHO}] \text{----------} 5.2$$

where k_{obs} = Pseudo- first order rate constant and is expressed in this study and

C is a constant.

[HCHO] = Concentration of formaldehyde,

k_2 = true second order rate constant.

The Pseudo – First and second order plots for the reaction of Plasma Albumin and Formaldehyde in water and ethanol-water mixtures at constant conditions of formaldehyde and plasma albumin concentrations at human body temperature showed pseudo –first order and over all second order with respect to both formaldehyde and plasma Albumin in both media with mean rate constants of 0.099 (±0.58) and 0.06 ±0.028) $dm^3\ mol^{-1}\ s^{-1}$ respectively.

The results of the pseudo-first order and second order rate constants for the plasma albumin and formaldehyde reaction in water show a mean second order rate constant of 0.099 (±0.58) $dm^3\ mol^{-1}\ s^{-1}$ from the varying formaldehyde concentrations of between 0.27×10^{-1} - 1.0×10^{-1} mol dm^{-3} and a constant plasma albumin concentration of 0.51×10^{-3} mol dm^{-3} at the given constant conditions.

Based on the data obtained the equation of the reaction in water can be presented by equation (5.3).

$$\frac{-d[H_2C(OH)_2]}{dt} k_2[H_2C(OH)_2][PABNH_2] - - - - - - - - -5.3.$$

Where k_2 = 0.099 (±0.034) dm^3 mol^{-1}

Similarly the plot of log k_1 versus log [HCHO] in ethanol-water mixtures as given in Figures 4.12.also give linear dependence on the concentration of formaldehyde Based on the above, the rate equations for reaction in Ethanol- waters (1.0 - 25%) mixtures can be presented by equation (4.3).

$$- - \frac{-d[H_2C(OH)_2]}{dt} = k_2[C_2H_5OCH_2(OH)][PABNH_2] - - - -5.4$$

where k_2 = second order rate constant calculated was constant and the average value in ethanol-water mixtures was k_2 = 0.06 (±0.028) dm^3 mol^{-1} s^{-1}.

The results also show that the reaction in water took place within 45 seconds and its rate increased with increase in the concentration of formaldehyde. This closely resembles its analogous member, glutaraldehyde which has similar chemistry. Glutaraldehyde has been reported to reacts readily with various proteins in solution (Hopwood, 1970). With high concentrations of both formaldehyde and glutaraldhyde, the solutions become yellow and many proteins form a gel. At low concentrations the reactions have been reported to be followed by the changes in the u.v. spectrum between 250 and 300 nm. A similar observation was found for formaldehyde at between 220 – 250 nm and at 400nm. Both the kinetics have been found pseudo-first-order. The activation energies for the reactions between proteins and glutaraldehyde were reported to be about 46.02 kJ mol^{-1} and the rates of reactions were found to increase with pH .This according to the authors suggests

that the proteins have not been denatured to any marked extent by the glutaraldehyde fixation. The rate of formation of glutaraldehyde-protein links per protein molecule glutarated was also found to be approximately 1.0sec^{-1} mol^{-1} (Hopwood, 1970). In contrast the activation energy for the reaction of formaldehyde with plasma albumin at room temperature, 25 ^{0}C have been found in a preliminary study to be 1.866 J mol^{-1} and a rate constant of 1.117 (±0.58) $dm^3mol^{-1}sec^{-1}$ for the plasma albumin-formaldehyde crosslink. The stoichiometric studies showed that two moles of formaldehyde react with one mole of plasma albumin. A stoichiometry of 1:1 has been reported for permanganate and formaldehyde (Singh, 1968).

The Pseudo – First and second order plots for the reaction of Plasma Albumin and Formaldehyde in Water and ethanol-water mixtures at constant conditions of formaldehyde and Plasma albumin concentrations at human body temperature showed Pseudo – First order and over all second order with respect to both formaldehyde and plasma Albumin in both media with mean rate constants of 0.099 (±0.58) and 0.06 ± 0.028) dm^3 mol^{-1} s^{-1} respectively. The proposed rate laws are as follows:

$$\frac{-d[H_2C(OH)_2]}{dt} k_2[H_2C(OH)_2][PABNH_2] - - - - - - - - - - 5.3$$

$$\frac{-d[H_2C(OH)_2]}{dt} = k_2[C_2H_5OCH_2(OH)][PABNH_2] - - - - - - -5.4$$

k_2 = 0.099 (±0.034) dm^3 mol^{-1} s^{-1} in water

k_2 = 0.06 (±0.028) dm^3 mol^{-1} s^{-1} in ethanol-water mixtures.

5.5.4 Ionic strength studies on the reaction between plasma albumin and formaldehyde in water and 15% ethanol-water mixture

The results of the ionic strength studies on the reaction of plasma Albumin with formaldehyde in water solution and ethanol-water mixtures show that there is a linear rate dependence on the ionic strength of medium and the rate increases with increase in the ionic strength of the salt. The plots of logk_2 versus $I^{1/2}$ were linear in both water solution and ethanol-water mixtures. The rate increased with increase in the ionic strength of the salt in water but decreased in 15% Ethanol - water mixture. Higher concentration of inert ions in solution shields the ions in the equilibrium with counter cations ions and counter anions in their ionic atmosphere. If solubility increases with ionic strength , then concentration of the inert ions increases hence activity coefficient decreases. For those reactions in which neutral species are present and for which the product of charges will be zero there effect will not be felt but that of the total counter ions which decreased the rate of reaction with their concentrations.It is also reported that, ionic strength is a factor of the ionic valence squared. Thus, monovalent ions such as $Na^{+,}$ K^{+}, Cl^{-}, and Br^{-} contribute to the ionic strength as a function of their respective concentrations. However, even trace levels of polyvalent ions such as Mg^{2+}, Ca^{2+}, and Al^{3+} can increase the ionic strength of the system disproportionately. It is for this reason why many wastewater treatment facilities use $FeCl_3$ as a flocculant (Olson, 1997).

This is in agreement with the study of Singh *et al.*, (1968) who found that formaldehyde in solution exist in a hydrated form which later dissociate to form a negative charge intermediate that could react with the added ions. Generally the rate of reaction basically is found to depend on the product of charges of ions present in a solution. Since the

compounds dissociate in a solution and forms cations and anions, so their product matters a lot. If the product of charges is positive, then the rate of reaction will increase automatically, the higher the product the faster will be the rate. Similarly, if the product of ions present in a solution is negative then the rate of reaction will decrease. For those reactions in which neutral species are present and for which the product of charges will be zero, there will be no effect on the rate of reaction. In this study, the species are neutral so added anions and cations that provide the ionic strength effects. The reason for the decrease is therefor due to the greater influence of ionic orientation for regulating the mireaction velocity. Favourable ionic interactions have been found to important for binding of biomolecular specie. A study by Sem and Kasper (1995) found that the results were consistent with favorable ionic interactions being important for binding NADPH and cytochrome C and with product ($NADP^+$) release becoming the rate-limiting step.

5.5.5 Temperature dependence studies in water and ethanol-water mixtures

The results of the temperature studies in water and ethanol- water systems as presented ealier showed a second order rate constant but unlike in most reactions in which the rate increases with temperature, the increase was not sharp this could be due to the denaturation of the plasma albumin substrates which are responsible for the reaction, hence decrease in the concentration of the plasma albumin.

On the basis of collision and transitional theory, the Arrhenius and Erying plots were used to calculation parameters. From the results, the negative enthalpy and entropy of activation values ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) parameters indicated non favorable thermodynamic

equilibrium of the solvent molecules in activated state, that is the reaction passed through an ordered transition state (Asadi *et al*, 2010) From the results from typical linear Arrhenius and Erying plots as given in Figures 4.16 - 4.21 and in Tables 4.14 - 4.19, the negative $\Delta S^{\neq}$ parameters as shown indicated random distribution of the solvent molecules in activated state that is the reaction passed through an ordered transition state (Asadi *et al*, 2010). The negative activation (Ea = -0.00807 kJmol^{-1}**)** and negative values of $\Delta H^{\neq}$, $\Delta \mathbf{S}^{\neq}$ but a small positive $\Delta G^{\neq}$ clearly showed that the reaction is not spontaneous in aqueous solution over the considered temperature range. However from the equation of state the value of the universal gas constant at body temperature, RT (2.577 kJ mol^{-1}) >> 0.07 kJ mol^{-1} and more than compensate for the Gibs free energy of activation barrier that is positive hence the driving force for the reaction to take place may be by collision or transition /activated complex mechanisms.

However it is found that it is possible to have a negative activation energy values when rates of reaction decreases with increase in temperature. Reactions exhibiting these negative activation energies are reported typically barrierless reactions, in which the reaction proceeding relies on the capture of the molecules in a potential well. Increasing the temperature leads to a reduced probability of the colliding molecules capturing one another with more glancing collisions not leading to reaction as the higher momentum carries the colliding particles out of the potential well. This is usually expressed as a reaction cross section that decreases with increasing temperature. Such situation no longer leads itself to direct interpretations as the height of a potential barrier. Suffix to

say that if a positive activation energy keeps a reaction from occurring until that amount of energy is provided from the environment then a negative activation energy would imply that a reaction could not stop from occurring once started.. The reactants already have plenty of energy for the reaction so the only way to prevent the reaction would be to keep the reactants apart. If this energy is negative then you would say that this is a barrier-less reaction and the reaction rate is proportional to the collision frequency (Jensen, 2010). As reactions were carried out in water and ethanol-water mixtures it indicates that the reaction is not favoured at high temperatures in the two systems.. Also as observed earlier, the reactions were mostly completed within 45 seconds since the thermodynamic properties of the activated complex are usually taken as a measure of the solution effects (El-Mallah *et al.,* 2010)..

5.5.6 The effect of hydrogen ion concentration on the reaction Rate

The rate constants of the reactions in water and ethanol – water systems increased with decrease in pH. This shows that the rate constants were found to be pH dependent in the range investigated. The rate constants of the reactions in water and ethanol-water systems increased with decrease in pH. This shows that the rate constants were found to be pH dependent in the range investigated. This is in agreement with the literature report that reactions of formaldehyde with amino acids and amines base catalysed and favoured in alkaline conditions (bedino,2003). A plot of $\ln k_2$ versus $[H^+]$ gave a linear graph of slope , 0.103 with a correlation coefficient of 0.907.

The acid dependent rate constant for reaction in water system can be represented by equation (5.5) (Li *et al*., 2007)

$-d[PABNH_2]/dt = a + b[H^+][PABNH_2][HCHO]$---------- 5.5

where 'a' = -4.262

'b'= 0.103 dm^6 mol^{-1} s^{-1}

5.5.7 Effect of added anions and cations on the reaction rate

As ealier observed ,the effect of added anions (CO_3^{2-} *and* NO_3^-) and cations (Fe^{3+}, Ca^{2+} and Zn^{2+}) in the range 0.05 - 1.0 mol dm^{-3} were found to enhanced the reaction in the water Generally the rate of reaction basically is found to depend on the product of charges of ions present in a solution. Since the compounds dissociate in a solution and forms cations and anions, so their product matters a lot. If the product of charges is positive, then the rate of reaction will increase automatically, the higher the product the faster will be the rate. Similarly, if the product of ions present in a solution is negative then the rate of reaction will decrease. For those reactions in which neutral species are present and for which the product of charges will be zero, there will be no effect on the rate of reaction. This assertion is not however valid in species in solution. Roses *et al*, (1996) evaluated the dependence of the holdup time of the ionic species on the mobile phase properties (pH, solvent composition, and ionic strength) was evaluated. Their study demonstrated that the hold up time of the neutral species does not depend on the mobile phase properties, but the hold up time of the ionic species depends on the particular buffer used.

5.5.8 Biochemical reaction mechanisn of formaldehyde – plasma albumin adduct

The findings from this study have shown that the prepared adduct exhibits glycoprotein properties of an aldehyde or Mannan- binding lectin and is a schiff base. It can react in a similar way just as the in vivo non-enzymatic reaction between reducing sugars and proteins in glacosylation (the covalent bonding of blood glucose to the red blood cells). Lectins are glycoproteins of plants and animals origin that recognize and bind to specific oligosaccharides. Common examples of lectins include; Concanavalin A and wheat germ agglutinin which are plant lectins. The C-type lectin-like domain which is a Ca^{2+} binding carbohydrate recognition domain in many animal lectins,; the CHO recognition and /binding moiety of glycol proteins, glycolipids and proteoglycans, animal lectins. The aldehyde recognition and binding lectins are reported to be a factor in: cell-cell recognition, adhesion of cells to the extra cellular matrix, interaction of cells with chemokines and growth factors. They also acts in recognition of disease-causing micro organisms, initiation and control of inflammation (Peumans and VanDamme., 1995).

Other examples of animal lectins include Mannan-binding lectin (MBL) which is a glycoprotein found in blood plasma. It binds cell surface carbohydrates of disease-causing micro-organisms and promotes phagocytosis of these organisms as part of the immune response (Diawan, 2008). Human blood proteins like hemoglobin and serum albumin are else where reported to undergo slow non - enzymatic glycation with oxidizing agents such as formaldehyde, mainly by forming schiff bases between α - amino groups of lysine and sometimes arginine and glucose molecules in the blood (Rajbar 1968) and elevated glycol albumin has been reported in diabetes mellitus (Iberg *et al.* 1986).

Thus the mechanism of the antimicrobial activity of the prepared adduct and formaldehyde in particular in the human body may be said to be by covalent binding by the Mannan or aldehyde aldehyde group to sugar moieties in cell walls or membranes and thereby changing the physiology of the membrane to cause

agglutination, mitosis, or other biochemical changes in the cell (Swanson *et al.*, 2010). This adduct may also undertake the non-enzymatic glycation which might lead to human health complications and diseases such as diabetes (Al-Abed *et al.*, 1999; Stitt, 2001). Glycation is reported to occur via the maillard reaction in which a reducing sugar reacts with an amino group on a protein, either at the NH_2 terminus or at the e-amino group of lysine residues (Tagami *et al.*, 2000; Yeboah *et al.*, 2004).

5. 5. 9 Kinetic study of reactions of plasma albumin with formaldehyde in ethanol - water mixtures and water solution using Bronsted - type plot

The pKa values which were obtained in this study show that the values for the plasma albumin in ethanol-water mixtures and in water ranged from 5.5 – 8.0 tending towards the alkaline region. The result also suggest that the amino acids involved in the reactions are Histdine and terminal α - amino acids. The results are in quite agreement with reported literature values for amino acids side chains which are common in plasma albumin and tends to be in the pK range of 3.75 to > 12 (Tong, 2003 and Berg *et al,* 2002) The Brønsted-type plot for the reactions of plasma albumin with formaldehyde adduct in water solution and ethanol-water mixtures were found to be linear with β = 0.071 and 0.0195 respectively. The reaction mechanisms that have these low values of proportionality constants α or β are considered to have a transition state closely resembling the reactant with little proton transfer (Cox *et al.*, 1988).Thus, one might suggest that the cross-linking of formaldehyde with plasma albumin in ethanol-water mixtures and in water medium proceed through transition state which have low values for linear Bronsted constants β and could be considered to have a transition state closely resembling the reactant with little proton transfer and base catalysis.

The results of the Bronsted plots of $\log k_2$ vs pka for plasma albumin – formaldehyde adduct in both ethanol-water mixtures and water solution are presented in Figures (4.38 -

4.39). The results show the β_{nuc} slope values of $\beta = 0.071$ and 0.0195 respectively. According to Cox *et al.,* (1988), the linear free energy relationships have proven to be valuable tools in studies of reaction mechanisms, in particular in determining the changes in transition state (TS) structure due to partial bond formation or secession and the magnitude of β_{nuc} values has been used as a measure of reaction mechanism. If proton transfer is complete in the transition state then the change in the free energy of the transition state should be the same as the change in free energy for the reference reaction; slope = 1, (Cox *et al.,* 1988; Mihai *et al.*, 2003).

The workers also noted that the Brønsted equation as a free – energy relationship also implies that the Gibbs free energy for proton dissociation is proportional to the activation energy for the catalytic step. When the relationship is not linear, the chosen group of catalysts do not operate through the same reaction mechanism. The reaction mechanisms that have low values for proportionality constants α or β are considered to have a transition state closely resembling the reactant with little proton transfer while those with a high value indicate that the proton transfer in the transition state is almost complete (Cox *et al.*,1988). The Bronsted slope is also reported to be a rough measure of the extent of proton transfer at the transition state: the extent to which the transition state respond to a change in catalyst relative to the change in the reference reaction;is as follows: value of α or $\beta = 1.0$ indicate that proton is fully transferred; value of or $\beta = 0.0$ indicate that proton is not transferred at all and values α or β between 0.0-1.0 indicate that proton is in "flight" at the transition state (Mihai *et al.,* 2003). Linear Brønsted-type plot with

βnuc = 0.88 in the pKa range 5.95 - 11.02 were concluded to proceed through a stepwise mechanism in which breakdown of the intermediate is the rate determining step. On the other hand, reactions which proceed through a concerted mechanism have often been reported to result in a β_{nuc} value of 0.5 ± 0.1 (Shin *et al*., 2008).

5. 5. 10 Mechanistic proposals of the rate of reaction of formaldehyde with plasma albumin in water

In neutral or alkaline solutions , the amino groups or proteins were reported to react in a readily reversible manner with two moles of formaldehyde, with addition of each and apparent lowering in pH. The resulting displacement of the acid equilibrium by formaldehyde is said to be the basis of the formol titration (Gary and Robert, 1971).

. In this study the reaction of formaldehyde with plasma albumin,showed that the stoichiometric ratio of formaldehyde with plasma albumin in aqueous medium was 1:12 and is pseudo-first order with respect to formaldehyde and over all second order at constant conditions, $I^{1/2}$ = 0.5 mol dm^{-3}, λmax = 255nm. Based on the results the mechanisms for the reaction of formaldehyde with plasma albumin in aqueous solution was proposed as given in equation (5.6 – 5.30).from the overall equation of reaction:

$$H_2C(OH)_2 + 2PABNH_2 \rightarrow PABNHCH_2NH\,PAB - 5.1$$

$$H_2CO + H_2O \rightleftharpoons H_2C(OH)_2 \quad ---fast\ equibrium----- 5.6$$

$$H_2C(OH)_2 + PABNH_2 \xrightarrow{k_2} PABNHCH_2OH + H_2O --slow---- 5.7$$

$$PABNHCH_2OH + PABNH_2 \xrightarrow{k_3} PABNHCH_2H\,N\,PAB + H_2O - slow - 5.8$$

The slow step determines the rate , therefore we have two sets of mechanisms.

First slow step:

We consider the first slow step equation (5..7)

Rate law = $k_2[H_2C(OH)_2][PABNH_2] - - - - - 5.9$

At steady state

$$\frac{-d[H_2C(OH)_2]}{dt} = k_1[H_2CO][H_2O] - k_{-1}[H_2C(OH)_2] + k_2[H_2C(OH)_2][PABNH_2]$$

$$= 0 - 10$$

$$k_1[H_2CO][H_2O] = H_2C(OH)_2[k_{-1} + k_2][PABNH_2] - - - - - - - - 11$$

$$H_2C(OH)_2 = \frac{k_1[H_2CO\ ]\ [H_2O]}{k_{-1} + k_2[PABNH_2]} - - - - - - - - - - 12$$

Substituting in equation [12] in the rate equation, 5.9

$$Rate = \frac{k_1k_2[H_2CO][H_2O][PABNH_2]}{k_{-1} + k_2[PABNH_2]} - - - - - - - - - - - 13$$

(i). If $k_{-1>>>}\ k_2[PABNH_2]$, then

$(k_{-1} + k_2[PABNH_2]) = k_{-1}$ - ----------14

$$Rate = \frac{k_1k_2[H_2CO][H_2O][PABNH_2]}{k_{-1}} - - - - - - - - - 15$$

$$Rate = Kk_2[H_2CO][H_2O][PABNH_2] \text{ ----------16}$$

where $K = k_1/k_{-1}$

$$Rate = k'[H_2CO][PABNH_2] \text{ ----------17}$$

where $k' = Kk_2$

The reaction is therefore first order with respect to each of the reactants and over all second order

(ii). If $k_{-1} <<<< k_2[PABNH_2]$, then

$\{ k_{-1} + k_2[PABNH_2] = k_2[PABNH_2]$ ----------18

$$Rate = \frac{k_1 k_2 [H_2CO][H_2O][PABNH_2]}{k_2[PABNH_2]} \quad \text{---------- 19}$$

$Rate = k_1[H_2CO]$,---------- 20

The reaction is pseudo - first order with respect to H_2CO

Second slow step:

We consider the second slow step equation (5.8)

$$PABNHCH_2OH + PABNH_2 \xrightarrow{k_3} PABNHCH_2NH\,PAB + H_2O - \text{slow } 5.8$$

Rate law = $k_2[[PABNHCH_2OH]\ [PABNH_2]$ ----- 5.9

At steady state

$$\frac{-d[H_2C(OH)_2]}{dt} = k_2[\ H_2C(OH)_2][PABNH_2] - k_{-2}[PABNHCH_2OH][\ H_2O] + k_3[PABNHCH_2OH][PABNH_2] \quad \text{-- ----5.21}$$

$$k_2[\ H_2C(OH)_2][PABNH_2] = PABNHCH_2OH\ [[k_{-2}[\ H_2O] + k_3[PABNH_2] \quad --5.22$$

$$PABNHCH_2OH = \frac{k_2[H_2C(OH)_2[PABNH_2]}{k_{-2}H_2O + k_3[PABNH_2]} \quad 5.23$$

Substituting equation [5.23] in the rate equation, 5.9

$$Rate = \frac{k_2 k_3[\mathrm{H_2}C(OH)_2[PABNH_2]\,[\,PABNH_2]}{k_{-2}H_2O + k_3[PABNH_2]} - - - - - - \; 5.24$$

$$Rate = \frac{k_2[\mathrm{H_2}C(OH)_2[PABNH_2]}{k_{-2}H_2O} - - - - - - 5.25$$

$$Rate = \frac{K[\mathrm{H_2}C(OH)_2[PABNH_2]}{H_2O} - - - - - - 5.26$$

The reaction is first order with respect to methylglycol adduct and plasma albumin and overall second order but inversely related to concentration of water.

5.5.11 Mechanistic proposal of the rate of reaction of formaldehyde with plasma albumin in ethanol - water medium

Aldehydes are known to under go addition reaction with a an equivalent of alcohol to form a tetrahedral intermediate, a hemiacetal and finally to acetal in the presence of additional mole of alcohol. The reaction is known to be both acid and base catalysed (Bruise, 2004). Similarly, Austin and Fraekel-Conrat, (1992) reported that primary amino groups (-NH_2) such as N-terminal and lysine α - amino groups of proteins, are known to react rapidly with the simple members of aldehyde group to form Schiff bases – N=$CHCH_3$. The Schiff bases are however found to be unable to react further with alcohols. These workers however found that just like some previous work showed, the exocyclic amino groups of nucleic acid components react quickly at ambient temperature with acetaldehyde and ethanol to yield mixed acetals [R-NH-$CH(CH_3)$-O-C_2H_5]. Their work found that the same type of reaction occurs readily with the nitrogen of 3-substituted indoles (e.g., indole-3-acetic acid and N-acetyltryptophan), analogues of the amino acid tryptophan. In relation to the above report, we found in this study that the rate

of the reaction of formaldehyde with plasma albumin in ethanol-water mixtures decreased with increase in the percentage ethanol – water mixtures. The mechanism for the plasma Albumin reaction with formaldehyde in ethanol – water mixtures was then proposed as given in the equations (5.27 - 5.37) below:

$$H_2CO + H_2O \rightleftharpoons H_2C(OH)_2 \quad ---fast\ equibrium---- 5.6$$

$$H_2C(OH)_2 + C_2H_5OH \xrightarrow{k_1} C_2H_5OCH_2(OH) --fast-- 5.27$$

$$C_2H_5OCH_2(OH) + PABNH_2 \xrightarrow{k_2} PABNHCH_2OC_2H_5 ---slow--- 5.28$$

The slow step is the rate determining step

$$Rate = k_2[C_2H_5OCH_2(OH)][PABNH_2] ---------- 5.29$$

At steady state

$$\frac{-d[C_2H_5OCH_2(OH)}{dt} =$$

$$k_1[H_2C(OH)_2][C_2H_5OH] - k_{-1}[C_2H_5OCH_2OH] + k_2[C_2H_5OCH_2OH][PABNH_2] = 0 --5.30$$

$$k_1[H_2C(OH)_2][C_2H_5OH] = C_2H_5OCH_2OH][k_{-1} + k_2[PABNH_2]] --- 5.31$$

$$\frac{k_1[H_2C(OH)_2][C_2H_5OH]}{k_{-1} + k_2[PABNH_2]} = [C_2H_5OCH_2OH] --- 5.32$$

Substituting equation (5.30) into the rate raw

$$Rate = \frac{k_1k_2[H_2C(OH)_2][C_2H_5OH][PABNH_2]}{k_{-1} + k_2[PABNH_2]} ----- 5.33$$

When

$k_{-1} >>>>> k_2[PABNH_2]$ **then**

$k_{-1} + k_2[PABNH_2]$ **reduces to** k_{-1}

$$Rate = \frac{k_1[H_2C(OH)_2][C_2H_5OH]}{k_{-1}} \text{ – – – – – – – } 5.34$$

$$Rate = \frac{k_A[H_2C(OH)_2][C_2H_5OH]}{} \text{ – – – –} 5..35$$

Where k_A is the apparent second order rate constant.

The reaction is first order with respect to $H_2C(OH)_2$ in ethanol

When $k_{-1} <<<<< k_2[PABNH_2]$ **then** $k_{-1} + k_2[PABNH_2]$ **reduces to** $k_2[PABNH_2$

$$Rate = \frac{k_1 k_2[H_2C(OH)_2[C_2H_5OH][PABNH_2]}{k_2[PABNH_2]} - -5.36$$

$$Rate = k_1[H_2C(OH)_2][C_2H_5OH] \text{ – – – – –} 5.37$$

The reaction is first order with respect to methylglycol in ethanol

5.5.12 Mechanistic proposals for ethanol-water mixtures based on SESMORTAC model

In this study the rate of the reaction was found to decrease with increase in the percentage ethanol – water mixtures while rate of the reaction in water solution was found to increase with increase in the water. Based on the findings of Babak *et al*, (2005), the two proposed mechanisms in water solution and ethanol-water mixtures could be said to involve two forms. The first mechanism occurs in pure water and the related rate equations (5.38 - 5.44) as follows:

Rate = k_2 [H_2C $(OH)_2$][$PABNH_2$].

Where k_2 is the observed rate constant in water.

The second mechanism could be written as follows: Adding Ethanol to the reaction medium results in formation of ACSM and decreases the rate of the reaction.

$$[(PABNH_2 + H_2C(OH)_2).nC_2H_5OH]cage + H_2O \xrightleftharpoons[k_{-1}]{k_1}$$

$$[(C_2H_5OCH_2OH).nH_2O]cage + nC_2H_5OH \xrightarrow{k_2} PABNHCH_2OC_2H_5 \quad \text{-------5.38}$$

$\|\text{ACSM}\|$

Where ethanol molecules are shown by $n$$C_2H_5OH$ and k_1 , k_{-1} and k_2 are the fundamental rate constants of the reaction of formaldehyde with plasma albumin in ethanol- water mixtures. Assuming that at a steady- state concentration of ACSM is attained in the mixed solvent, we have

$$[ACSM] = \frac{k_1[PABNH_2][H_2C(OH)_2][H_2O]^n}{k_{-1}[C_2H_5OH]^n} \text{ – – – – – – – – – – 5.39}$$

and the rate equation is $v = k_2[ACSM]$ – – – – – – – – 5.40

by substituting (5. 37) in (5..38) we have

$$v = \frac{k_2k_1[PABNH_2][H_2C(OH)_2][H_2O]^n}{k_{-1}[C_2H_5OH]^n} \text{ – – – – – – – – – –} \quad 5.41$$

thus the rate changes directly with $[\ H_2O]^n$ and inversely with $[C_2H_5OH\ ]^n$

The experimental rate equation of plasma albumin reaction with formaldehyde in ethanol - water was found to be

$v = k_{obs}[H_2C(OH)_2][PABNH_2]$ – – – – – – – 5.42

by equating (5.39) and (5.40.) we have

$$k_{obs}[H_2C(OH)_2][PABNH_2] = \frac{k_2k_1[PABNH_2][H_2C(OH)_2][H_2O]^n}{k_{-1}[C_2H_5OH]^n + k_2} \quad \text{----- 5.}$$

$$k_{obs} = \frac{k_2 k_1 [H_2O]^n}{k_{-1}[C_2H_5OH]^n + k_2} \quad \text{----------5.44}$$

The reaction rate is lowered in solvent mixtures with high ethanol concentrations.

5.5.13 Mechanistic proposal based on bronsted-plot in water solution and ethanol - water-mixtures

The Brønsted-type plot for the reaction of plasma albumin with formaldehyde in water and ethanol-water mixtures have been found to be liner wiyh the β_{nuc} slope values of $\beta = 0.071$ and 0.0195 respectively. When the relationship is not linear, the chosen group of catalysts do not operate through the same reaction mechanism. The reaction mechanisms that have low values for proportionality constants α or β are also considered to have a transition state closely resembling the reactant with little proton transfer while those with a high value indicate that the proton transfer in the transition state is almost complete (Cox *et al,* 1988). Linear Brønsted-type plot with βnuc = 0.88 in the pKa range 5.95 - 11.02 were concluded to proceed through a stepwise mechanism in which breakdown of the intermediate is the rate determining step. On the other hand, reactions which proceed through a concerted mechanism have often been reported to result in a β_{nuc} value of 0.5 ± 0.1 (Shin *et al*., 2008). Thus, we could suggest that the cross-linking reaction of formaldehyde with plasma albumin in ethanol-water mixtures proceeds through a proton transfer in the transition state that to is closely resembling the reactant with little proton transfer.

CHAPTER SIX

6.0 SUMMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

This study investigated the formaldehyde levels in some selected Cosmetics and other non- food materials as well as in human milk, urine, fish and milk products by Hantzsch spectrophotometric method using Acetylacetone and acetoacetanilide reagents, both ß-ketones. Eighteen (18) cosmetics and non-food samples namely: Napkin1,Napkin 2, Napkin 3, Lipstic1, Lipstic2, Lipstic3, Petals 1, Petals 2, Petals 3 Toilet tissue1, Toilet tissue 2, Toilet tissue3, Growers 1, Growers 2, Layers, Paint 1, Paint2, and Paint3 were analysed for formaldehyde level using both acetylacetone and acetoacetinilide reagents and the correlation between the results from the two methods were found to be significant at 95% confidence level with a ($p<0.0001$) indicating that the results from the two reagents were correlated. Similar approach was carried out using the acetoacetanilide, and acetylacetone reagents on (10) samples comprising of, fish, milk samples and body fluids. Breast milk (fresh milk) and urine samples, Fresh fish, Peak milk brand (liquid),

Cow milk (raw milk), Crown brand milk(powder), Cowbell brand milk (liquid and powder forms), Frozen fish, Dry fish, Smoked fish , Fried fish and Nunu brand milk(powder) were analysed. This result also indicate that the methods used are reproducible and suitable for the analysis of biological samples at rroom temperature.

The study also isolated plasma albumin from human blood samples using cold 90% v/v ethanol and 0.14 M HCl. The flocculent precipitate of the plasma albumin was dried over P_2O_5 to obtain a percentage recovery of 1.36 %. The dried isolated plasma albumin was further characterized for purity using protein analysis, optical rotation, and solubility and denaturation tests.

A 3,5-diphenyl-1, 4-dihydropyridine adduct was further prepared from acetoacetanilide a beta diketone, formaldehyde and plasma albumin and was characterized by measuring its melting point, IR analysis, Uv spectra characteristics as well as the microbial activity. The reagents were mixed in a one pot synthesis and were refluxed for one hour and allowed to stay overnight where a yellow adduct was obtained and was filtered and dried over phosphorus pentaoxide (P_2O_5) . An investigation of the kinetics of the reaction of formaldehyde with plasma albumin under normal and physiological conditions involving pH, temperature, reactants concentrations and ionic strength in water solution and (0 - 25%) Ethanol - water mixtures was also carried out. A rate constant of k_2 = 0.099 (±0.034) $dm^3\ mol^{-1}\ s^{-1}$ in water and k_2 = 0.06 (±0.028) $dm^3\ mol^{-1}s^{-1}$ ethanol-water mixtures respectively were obtained. A steady decrease in rate constant with decrease in dielectric constant was observed with statistically significant p-value of 1.000 on a two tailed correlation analysis at 95% confidence assurance.

The results show the plasma album contained 63.67% protein, with specific rotation of 8 at $[\alpha]_{598}^{32}$ at pH 7.2 and gradually dissolved in acidified water and ethanol. The plasma albumin was also found to be slightly turbid when dissolved in water indicating some degree of denaturation. The IR analysis showed the functional groups present in the adduct as follows: The band at 2900.76cm^{-1} was assigned to aldehydic C-H stretch and the one at 3845.5 cm^{-1} was found to be (O-H) stretch for water of hydration.

The sharp 2356.87 band was found to be due to methylene bridge CH_2 stretch in the adduct while the bands at 1647.2, 1517.4 cm^{-1} and 1032.63 cm^{-1} were assigned to N-H amines bend and C-N amines stretch respectively.

The adduct also showed some antimicrobial effect to some micro - organism such *as Bacillus subtilis* zone of inhibition 12.0 mm, *Proteum mirabilis*, zone of inhibition 16.0 mm and *Canidad albicans* zone of inhibition 14.0 mm. It was also found to have no effect on some fungi /algae such as *Escherichia coli* and *Staphylococcus aureus.*

The melting point analysis showed that theadduct had a melting temperature range of 179.5 - 180.4^{o}C. The adduct was found to absorbed ultra violet light at three maximum wavelengths, at max 250, 255 and 410 nm with an absorption coefficient of 1645, 1812 and 1483 L mol^{-1} cm^{-1} respectively. The findings from the study have shown that the prepared adduct was a Schiff base and a recognizing/ binding lectin type of aldehyde or mannan recognizing/binding moety of animal origin.

The kinetic study of the reaction of plasma Albumin with formaldehyde in water and ethanol-water mixtures showed that the reaction had a stoichiometric ratio of 1:2 and was pseudo-first order with respect to formaldehyde and overall second order with rate constants, k_2 = 0.099 (±0.58) $dm^3\ mol^{-1}\ s^{-1}$ in water and 0.06 ±0.028) $dm^3\ mol^{-1}\ s^{-1}$ in ethanol water system respectively and a half life of 5.92 and 11.55 seconds respectively. The results of the temperature studies in water and ethanol- water systems as presented ealier showed a second order rate constant but unlike in most reactions in which the rate increases with temperature, the increase was not sharp this could be due to the denaturation of the plasma albumin substrates which are responsible for the reaction, hence decrease in the concentration of the plasma albumin. On the basis of collision and transitional theory, the Arrhenius and Erying plots were used to calculate the parameters. From the results, the negative enthalpy and entropy of activation values ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) parameters indicated non favorable thermodynamic equilibrium of the solvent molecules in activated state, that is the reaction passed through an ordered transition state (Asadi *et al*, 2010) The negative $\Delta S^{\ddagger}$ parameters as shown indicated random distribution of the solvent molecules in activated state that is the reaction passed through an ordered transition state. The negative activation (Ea = -0.00807 $kJmol^{-1}$**)** and negative values of $\Delta H^{\neq}$, $\Delta \mathbf{S}^{\neq}$ but a small positive $\Delta G^{\ddagger}$ clearly showed that the reaction is not spontaneous in aqueous solution over the considered temperature range. However from the equation of state the value of the universal gas constant at body temperature, RT (2.577 kJ mol^{-1}) >> 0.07 kJ mol^{-1} and more than compensate for the Gibs free energy of activation barrier that is positive hence the driving force for the reaction to take place may be by

collision or transition /activated complex mechanisms A literature report ealier presented show that the negative $\Delta S^{\ddagger}$ value in water indicate that the reaction is compatible with the associative mechanism in this case a cross – linking / binding reaction as earlier reported in the literature.

The plasma albumin pKa values obtained in this study both in ethanol-water mixtures and water ranged from 5.5 – 8.0 tending towards the alkaline region. The result also suggest that the amino acids side chains involved in the reactions are Histidine or terminal α - amino acids. The corresponding values of the brønsted - type plots proportionality constants (β) for the reaction in water solution and ethanol-water mixtures were found to be 0.071 and 0.01 95 respectively. This shows that the reaction of formaldehyde with plasma albumin is based on binding or associative mechanism.

The results of this study have enabled us to verify that the alternative hypothesis holds. Some elevated values of formaldehyde were obtained in some of the selected foods, non-food products, human milk and urine samples that were analyzed for formaldehyde. Also, the reaction of formaldehyde with protein albumin in water and various ethanol water mixtures studied kinetically under various conditions similar to the human blood serum showed that formaldehyde binds plasma albumin in both aqueous and in ethanol-water mixtures slowly. The aldehyde functional group in the plasma albumin-formaldehyde adduct identified in the study established that the adduct has lectin properties which is associated with diabetes through advanced glycation products reaction. This has been found to be one of the health risks associated with the presence of formaldehyde in the human blood stream. These results also support the hypothesis that simple chemical reactions may lead to the adduction of nucleophilic macronucleus such as peptides or proteins. These reactions particularly, the Schiff base formation of aldehydes have

previously be shown to be capable of specifically interfering with costimulatory signaling on T cells yielding immunotoxic effects. Based on the data obtained, the mechanism of the reaction was proposed to be first order in formaldehyde and overall second order in water Similarly, the reaction in ethanol-water mixtures was found to be first order with respect to methylglycol. In general he reaction rates in both water and ethanol – water mixtures were found to be directly dependent on the concentration of the methylglycol in ethanol-water mixture but inversely related in water medium.

6.2 Conclusions

The results of the analyses and the correlation analysis showed that all the samples contained levels of formaldehyde. The results from the two methods were significant at 95% confidence level with a ($p<0.0001$) indicating that the results from the two reagents were correlated and suitable for the detection of biological samples. This is in agreement with the alternative hypothesis, H_1 which, states that elevated values of formaldehyde are present in commonly used foods, non food materials and human body fluids. The two results obtained were however insignificant compared to the reported toxic values of 0.8 gKg^{-1} HCHO for humans. Also, the reaction of formaldehyde with protein albumin in water and ethanol water mixtures in this study under various conditions similar to the human blood serum showed that one mole of formaldehyde binds two moles of plasma albumin forming Schiff bases slowly. The result also suggest that the amino acids side chains involved in the reactions are Histidine or terminal α - amino acids. The corresponding values of the brønsted - type plots proportionality constants (β) for the reaction in water solution and ethanol-water mixtures were found to be 0.071 and 0.01 95 respectively. This suggests that the reaction of formaldehyde with plasma albumin is based on binding or associative mechanism.

6.3 Recommendations

The reaction of formaldehyde with various components of the environment have been reported to be a significant risk factor to both animals and human health and result in amyloidal formations, fibrillations, diabetes, cancer and death in humans and constitutes a human risk factor. This study therefore recommends a continuous and routine analysis of formaldehyde in the environment. The reaction of formaldehyde with plasma albumin was also found to be based on binding or associative mechanism. We therefore recommend development of software and more funding of researches in these areas to add more insight in these common but complex health endangering chemical reaction.

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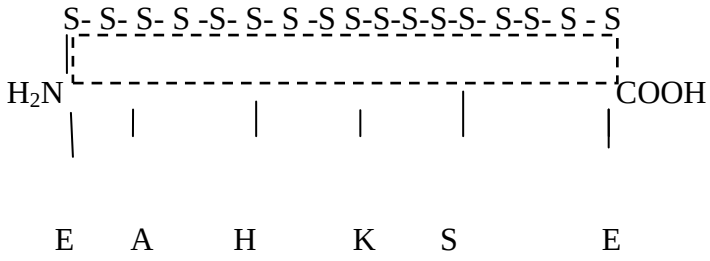
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LIST OF APPENDICES

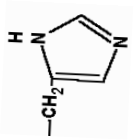
Appendix A: Schematic representation of the structure of plasma albumin



Where S-S= Disulphide bonds

E= Glutamic acid, Amino acid side chain = $HOOCCH_2CH_2CHCO_2H$(acidic)
NH_2

A= Alanine, Amino acid side chain= $CH_3CHCOOH$(neutral)
NH_2

H = Histidine Amino acid side chain= 

$H_2CHCOOH$ (basic)

$H_2NCHCOOH$

K= Lysine, Amino acid side chain= $CHCH_2CH_2CH_2CH_2NH_2$(basic)

S= Serine, Amino acid side chain= $HOCH_2$

$H_2NCHCOOH$(neutral)

E= Glutamic acid, Amino acid side chain = $HOOCCH_2CH_2CHCO_2H$(acidic)

NH_2

Appendix B: Properties of Intervening Sequence for the Structure of Human Serum

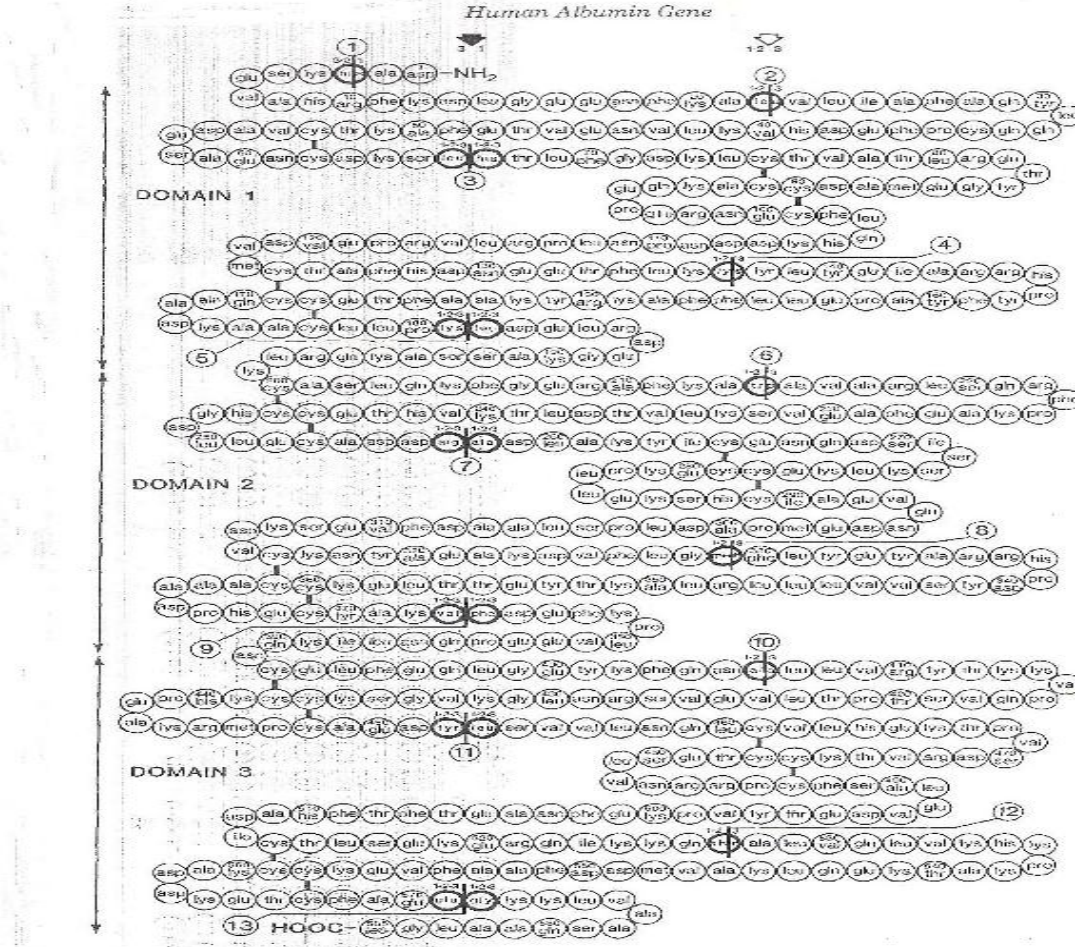
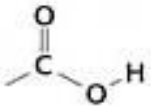
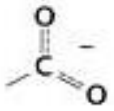
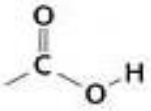
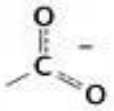
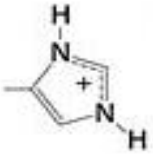
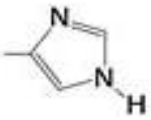
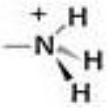
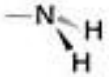
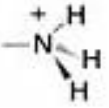
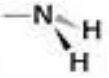
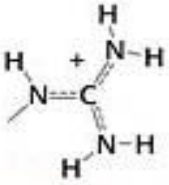
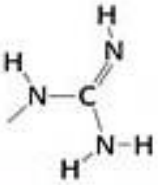


Figure 2.1 Position of intervening sequences within the structure of human serum albumin.

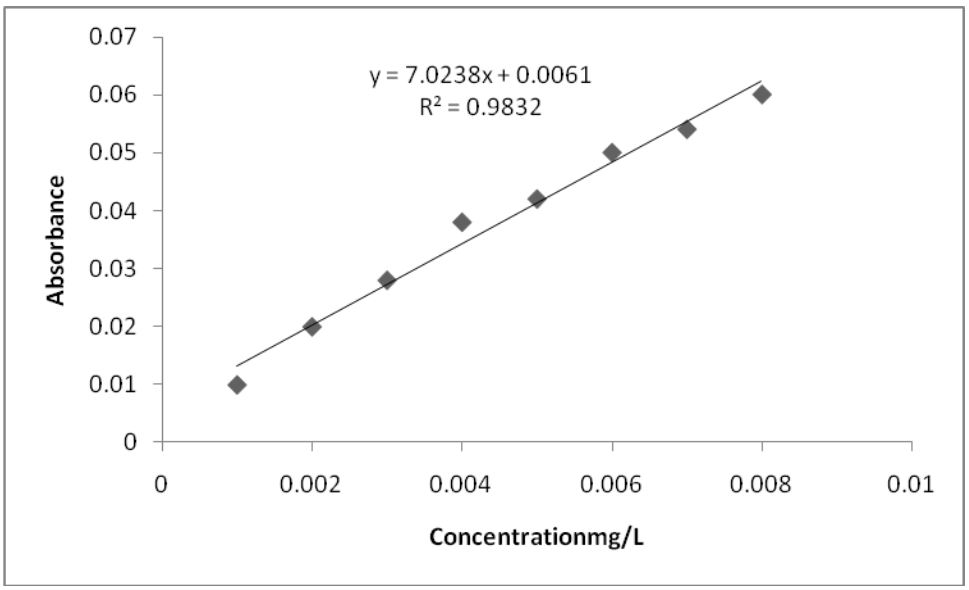
Appendix C: Side-chain pKa values in free amino acids

Group	Acid		Base	Typical pK_a
Terminal α-carboxyl group		⇌		3.1
Aspartic acid Glutamic acid		⇌		4.1
Histidine		⇌		6.0
Terminal α-amino group		⇌		8.0
Cysteine		⇌		8.3
Tyrosine		⇌		10.9
Lysine		⇌		10.8
Arginine		⇌		12.5

*pK_a values depend on temperature, ionic strength, and the microenvironment of the ionizable group.

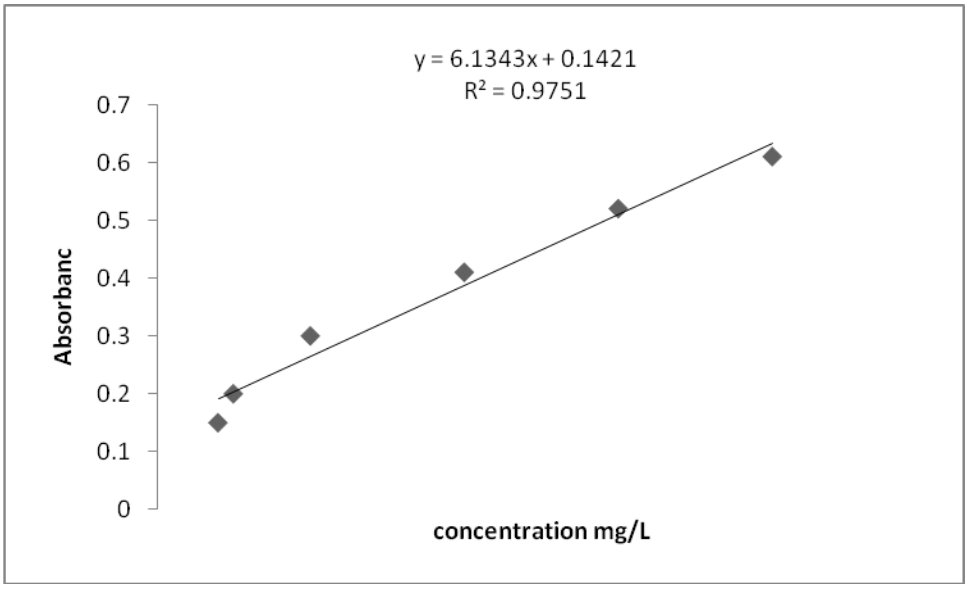
From (Berg *et al.*, 2002)

Appendix D: Calibration curve for determination of formaldehyde from samples using acetylacetone reagent at λ_{max} 410nm



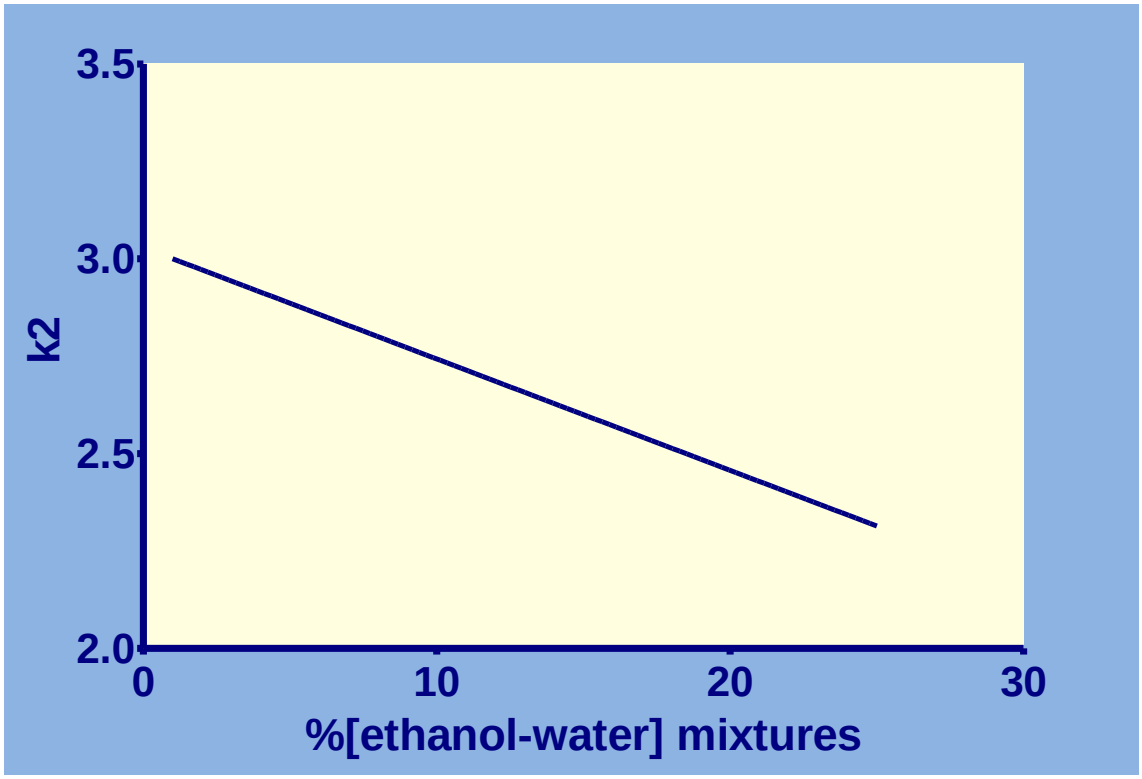
Absorbance	Concentration (mg/L)
0.01	0.001
0.02	0.002
0.28	0.003
0.038	0.004
0.042	0.005
0.05	0.006
0.054	0.007
0.06	0.08

Appendix E: Calibration curve for determination of formaldehyde from samples using acetoacetinilide reagent at λ_{max} 365 - 368nm



Absorbance	Concentration (mg/L)
0.15	0.08
0.2	0.01
0.3	0.02
0.41	0.04
0.52	0.06
0.61	0.08

Appendix F: Plot k_2 versus % ethanol-water mixtures



Appendix G: Table of values of time and absorbances for the determination of order and rate constants for reaction of plasma albumin and formaldehyde in water at constant conditions of formaldehyde and plasma albumin concentrations at human body temperature, [formaldehyde] = 2.7 x10^{-1}, [albumin] = 0.51x 10^{-3}, $I^{½}$ = 0.5 mol dm^{-3}, pH= 6.8, λ_{max} = 235nm and T= 37.0 ^{0}C

Time (s)	Absorbance	$(A_t - A_\infty)$	$\log(A_t - A_\infty)$
0	0.485	0.015	-1.824
5	0.483	0.013	-1.886
10	0.481	0.011	-1.959
15	0.479	0.009	-2.046
20	0.477	0.007	-2.155
25	0.474	0.004	-2.395
30	0.473	0.003	-2.523
35	0.472	0.00	-2.699
40	0.472	0.00	
45	0.472	0.00	

Appendix H: Table of values of time and absorbances for the determination of order and rate constants for reaction of plasma albumin and formaldehyde in water at constant conditions of formaldehyde and plasma albumin concentrations at human body temperature, [formaldehyde] = 3.0 x10^{-1}, [albumin] = 0.51x 10^{-3}, $I^{½}$ = 0.5 mol dm^{-3} , pH= 6.8, λ_{max} = 235nm and T= 37.0 ^{0}C

Time (s)	Absorbance	$(A_t - A_\infty)$	$\log(A_t - A_)$

0	0.569	0.014	-1.854
5	0.565	0.01	-2.000
10	0.563	0.008	-2.097
15	0.562	0.007	-2.155
20	0.561	0.006	-2.222
25	0.560	0.005	-2.301
30	0.558	0.003	-2.523
35	0.557	0.002	-2.699
40	0.556	0.001	-3.000
45	0.555	0.000	
50	0.555	0.000	

Appendix I: Table of values of time and absorbances for the determination of order and rate constants for reaction of plasma albumin and formaldehyde in water at constant conditions of formaldehyde and plasma albumin concentrations at human body temperature, [formaldehyde] = 6.0 x10^{-1}, [albumin] = 0.51x 10^{-3}, $I^{½}$ = 0.5 mol dm^{-3} , pH= 6.8, λ_{max} = 235nm and T= 37.0 ^{0}C

Time (s)	Absorbance	$(A_t - A_\infty)$	$\log(A_t - A_\infty)$

0	0.580	0.014	-1.854
5	0.578	0.011	-1.959
10	0.576	0.01	-2.000
15	0. 574	0.008	-2.097
20	0.572	0.006	-2.222
25	0.571	0.005	-2.301
30	0.569	0.003	-2.523
35	0.568	0.002	-2.698
40	0.567	0.001	-3.000
45	0.566	0.000	
50	0.566	0.000	

Appendix J : Table of Values of Time and Absorbances for the Determination of Order and Rate Constants for Reaction of Plasma Albumin and Formaldehyde in Water at Constant Conditions of Formaldehyde and Plasma Albumin Concentrations at Human Body Temperature, [Formaldehyde] = 7.0 x10^{-1}, [Albumin] = 0.51x 10^{-3}, I ^{½} = 0.5 mol dm^{-3} , pH= 6.8, λ_{max} = 235nm and T= 37.0 ^{0}C

Time (s)	Absorbance	$(A_t - A_\infty)$	$\log(A_t - A_\infty)$
0	0.579	0.014	-1.854

5	0.576	0.011	-1.959
10	0.573	0.008	-2.097
15	0.571	0.006	-2.222
20	0.570	0.005	-2.301
25	0.578	0.003	-2.523
30	0.577	0.002	-2.699
35	0.576	0.001	-3.000
40	0.575	0.00	
45	0.575	0.00	

Appendix K: Table of values of time and absorbances for the determination of order and rate constants for reaction of plasma albumin and formaldehyde in water at constant conditions of formaldehyde and plasma albumin concentrations at human body temperature, [formaldehyde] = 8 x10^{-1}, [albumin] = 0.51x 10^{-3}, I ^{½} = 0.5 mol dm^{-3} , pH= 6.8, λ_{max} = 235nm and T= 37.0 ^{0}C

Time (s)	Absorbance	(A_t- A_∞)	log(A_t- A_∞)
0	0.593	0.015	-1.824
5	0.591	0.013	-1..886

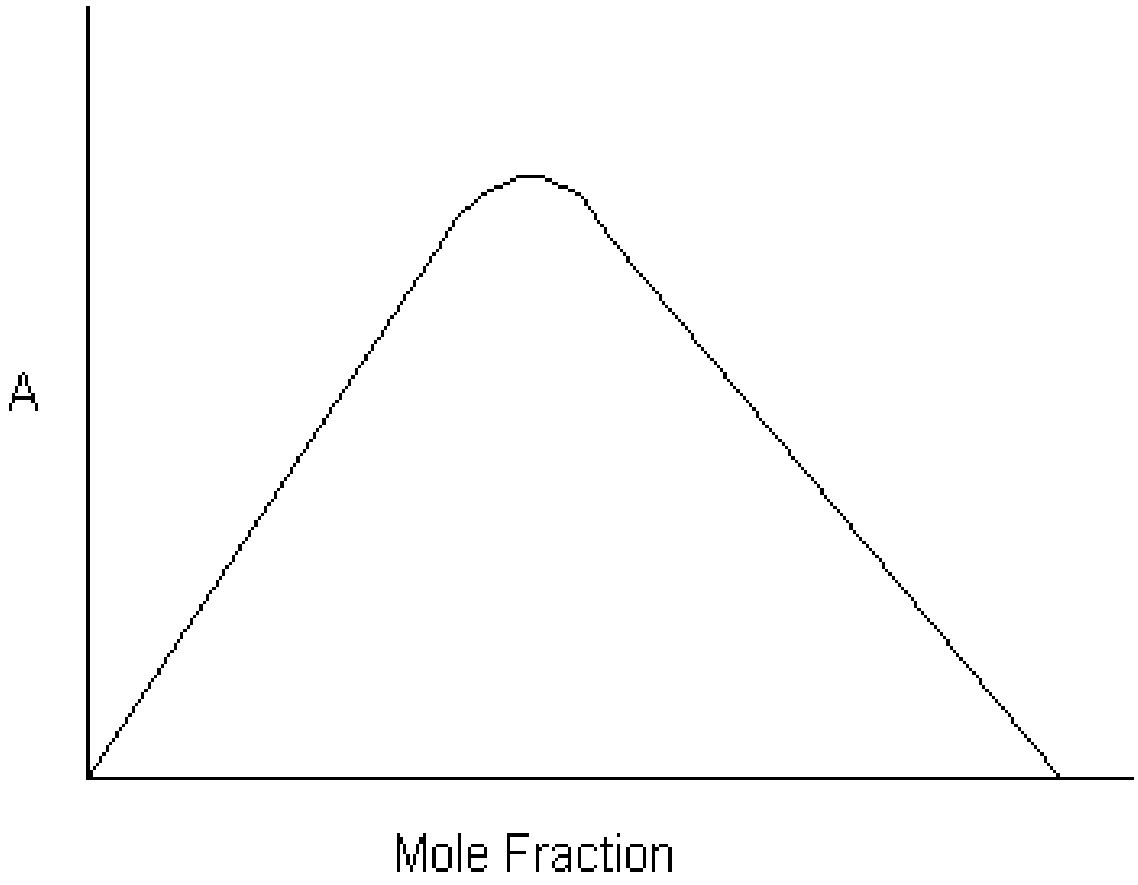
10	0.589	0.011	-1.959
15	0.587	0.009	-2.046
20	0.584	0.006	-2.222
25	0.583	0.005	-2.301
30	0.582	0.004	-2.398
35	0.581	0.003	-2.523
40	0.580	0.002	-2.699
45	0.579	0.001	-3.00
50	0.578	0.00	

Appendix L: Table of values of time and absorbances for the determination of order and rate constants for reaction of plasma albumin and formaldehyde in water at constant conditions of formaldehyde and plasma albumin concentrations at human body temperature, [formaldehyde] =10.0 x10^{-1}, [albumin] = 0.51x 10^{-3}, $I^{½}$ = 0.5 mol dm^{-3} , pH= 6.8, λ_{max} = 235nm and T= 37.0 ^{0}C

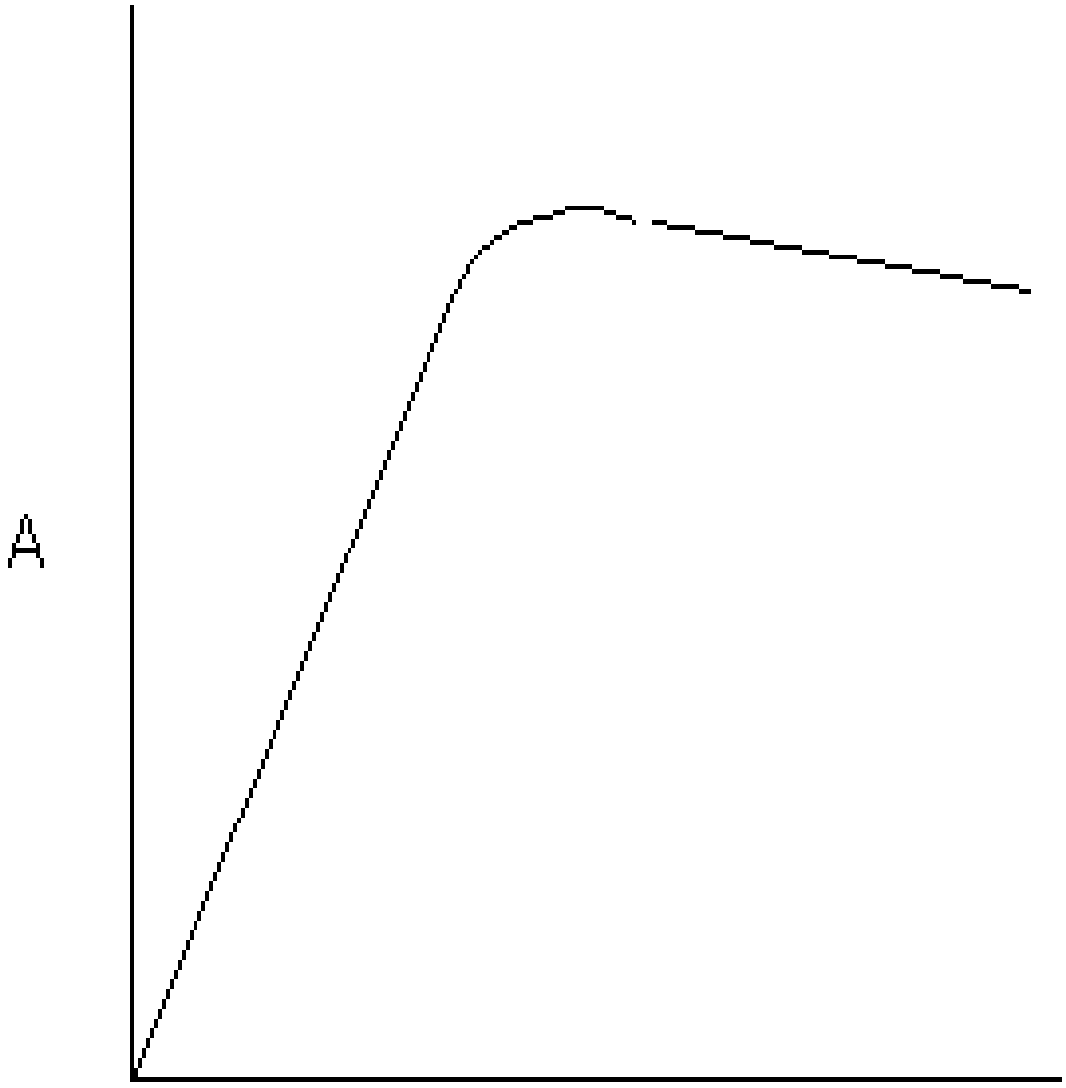
Time (s)	Absorbance	$(A_t - A_\infty)$	$\log(A_t - A_\infty)$
0	0.570	0.02	-1.699
5	0.568	0.018	-1.745
10	0.565	0.015	-1.824

15	0.561	0.011	-1.959
20	0.567	0.007	-2.155
25	0.566	0.006	-2.222
30	0.564	0.004	-2.398
35	0.576	0.002	-2.699
40	0.561	0.001	-3.000
45	0.560	0.000	
50	0.560	0.00	

Appendix M: A model plot of absorbance versus mole fraction as used in method of continuous variation (job's method).



Appendix N: A model plot of absorbanceversus mole ratio as used in mole ratio method


A
mol L/mol M+