THE EFFECT OF ANTIOXIDANTS ON THE *PARA*-AMINOBENZOIC ACID PHOTOSENSITISED FORMATION OF THYMINE DIMER AND SINGLET MOLECULAR OXYGEN

by

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Kwa wanangu wapendwa, Muhammad, Fatma, Hambal, Harith na Hudhaifah ningependa kuwakumbusha machache. Kwanza ni kuwa hakuna kiumbe aliyekamilika lakini uchaji Mungu ndiyo kilele cha hikma. Pili, subra ni ibada kama ibada nyenginezo. Mwisho ni kuwa "mvumilivu hula mbivu" na "Panapo nia pana njia."

ABSTRACT

On exposure to UV radiation DNA is damaged. The thymine cyclobutane dimer is one of the most significant of the lesions formed. This dimer is associated with the mutagenic and carcinogenic effect of UV radiation. It has been shown to cause skin cancer. Thymine dimerisation can be effected by direct irradiation or via an endogenous photosensitiser that is involved in energy transfer.

This deleterious effect of UV radiation has prompted the use of sunscreens as photoprotectants. *Para*-aminobenzoic acid (PABA) is an active ingredient that was once widely used in sunscreen formulations. Reports of allergic reactions and staining by PABA have led to the discontinuation of its use in sunscreen-containing products. It has been shown to be absorbed by human cells and to photosensitise the dimerisation of thymine in DNA. It can also photosensitise the formation of singlet oxygen. Singlet oxygen has been implicated in many photodynamic disorders, carcinogensis, free radical reactions and aging. Because the photochemical properties of PABA on DNA have been well established by a number of earlier researchers, it proved an appropriate choice of photosensitiser in order to determine the effect of vitamin antioxidants on the photosensitised formation of thymine dimer and singlet oxygen.

Vitamin E (α-tocopherol) and vitamin C (ascorbic acid) are two important anti-oxidants that have been extensively studied in free-radical reactions and cancer-related ailments. They are known to reduce the formation of thymine dimers and also quench singlet oxygen. They are found extensively in sunscreen formulations because of their photoprotective properties. The photoprotection is due to both their absorptive and antioxidant properties. They are known to act either individually or synergistically by physical quenching or reaction with free radicals. Their abilities to reduce the PABA-photosensitised formation of thymine dimer have been studied in this work. The study involved investigating their individual abilities and the synergism by varying their concentrations, the concentrations of thymine and PABA, and the photon flux.

The pH of the solutions was always maintained at 3.0. Analysis and quantification of the photoproducts was done by using reverse phase high performance liquid chromatography with a photodiode array UV detector.

Studies on the PABA-photosensitised production of singlet oxygen and its quenching by the antioxidants were also carried out at pH 3.0. With this regard, irradiation time and concentrations of PABA and the two vitamins were varied. Thymine was subsequently introduced so as to investigate its effect on singlet oxygen formation. The presence and change in concentration of singlet oxygen was monitored by using N,N-dimethyl-4-nitrosoaniline, RNO. The change in absorbance of RNO was measured at 350 nm, which is its maximum absorption wavelength at pH 3.0. The formation of singlet oxygen was inferred from these measurements.

PABA-photosensitised thymine dimer yield increased with an increase in irradiation time and an increase in the concentration of thymine. However, the yield decreased with an increase in the concentration of PABA. The presence of the vitamins C and E either acting alone or when combined had an effect on the yield of thymine dimer. Both the two antioxidants can increase or decrease thymine dimer yield. However, this depended on the concentrations of the reagents, the irradiation time and whether the two vitamins were in combination or alone. Therefore, both synergistic and antagonistic properties of the two vitamins were observed in the PABA-photosensitised thymine dimer formation.

Singlet oxygen formation generally increased with an increase in irradiation time except for the experiments in which the two vitamins were present together. When the two vitamins were present, there was an increase in the formation of singlet oxygen with irradiation time for a few minutes then the formation remained constant despite an increase in the irradiation time. An increase in the concentration of PABA also increased the formation of measurable singlet oxygen. The introduction of the antioxidants either individually or together decreased the yield of singlet oxygen. The presence of thymine had different effects on the total amount of measurable singlet oxygen

from those seen above. Generally, singlet oxygen formation increased with increase in concentration of thymine. Thymine in the presence of increasing concentrations of PABA showed different trends in the formation of singlet oxygen. These trends depended on the presence or absence of the antioxidants and whether the antioxidants were present individually or together. In this set of experiments, the presence of thymine resulted in an increase in the total amount of singlet oxygen formed. An exception to this is when vitamin E is introduced. In this case the presence of thymine indicated formation of a smaller amount of singlet oxygen. In both cases of the absence and presence of thymine, the trend in the formation of singlet oxygen with increase in concentration of any of the antioxidants varied depending on whether the antioxidant was alone or present with the other.

From the above it is evident that both synergistic and antagonistic properties of the two vitamins are exhibited regarding their ability to quench singlet oxygen in aqueous solution. The synergistic property of the two vitamins is lost in the presence of thymine. This implies that in the experimental conditions studied, thymine can photosensitise singlet oxygen formation despite the presence of the two vitamins. It is highly likely that both chemical and physical quenching by vitamins C and E take place. Some observations suggested a possible chemical interaction between the photosensitiser (PABA) and thymine with the quenchers. More work needs to be done in this regard to test this hypothesis.

PREFACE

This thesis presents work carried out by the author and has not been submitted in part, or in whole, to any other university. Where use has been made of the work of others it has been duly acknowledged in the text.

The experimental work described in this thesis was carried out in the School of Chemistry, University of KwaZulu-Natal, Howard College Campus, Durban from September 1999 to November 2004 under the supervision of Prof. B.S. Martincigh.

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Abbreviations

adenine	A
acetonitrile	ACN
cis-anti thymine dimer	*cis-anti*
cis-syn thymine dimer	*cis-syn*
cytosine	C
deoxyribonucleic acid	DNA
dimethylsulfoxide	DMSO
Escherichia coli	*E. coli*
Electron Spin Resonance	ESR
guanine	G
High Performance Liquid Chromatography	HPLC
Infrared	IR
lifetime	τ
Linear Combination of Atomic Orbitals	LCAO
Minimum Erythymal Dose	MED
methanol	MeOH
N,N-dimethyl-4-nitrosoaniline	RNO
Nuclear Magnetic Resonance	NMR
Octadecyl silane	ODS
Photodiode Array	PDA
photoreactivating enzyme	PRE
psoralen UVA	PUVA
pyrimidine-(6-4)-pyrimidone	(6-4)
quantum yield	φ
Reactive Oxygen Species	ROS
ribonucleic acid	RNA
semi-preparative	semi-prep
Sun Protection Factor	SPF
tetrahydrofuran	THF
thymine	T
thymine dimer	T<>T

thymine aggregate	T⌒T
trans-anti thymine dimer	*trans-anti*
trans-syn thymine dimer	*trans-syn*
uracil	U
vitamin C	Vit C
vitamin E	Vit E
wavelength	λ
wavelength of maximum absorption	λ_{max}
Xeroderma pigmentosum	XP

CHAPTER 1

INTRODUCTION

This thesis presents the results obtained from an investigation of the photochemical dimerisation of free thymine base in aqueous solution at pH 3.0 in the presence of the photosensitiser, *para*-aminobenzoic acid (PABA), and the effect of two antioxidants, α-tocopherol (vitamin E) and ascorbic acid (vitamin C), on this dimerisation. Results on the effect of thymine and the two antioxidants on the photosensitised production of singlet oxygen by PABA are also presented. The following introduction briefly reviews the photochemistry of deoxyribonucleic acid (DNA), the photochemical interaction between free thymine base and PABA, and possible effects of the introduction of the two antioxidants. It also discusses the photochemistry of singlet oxygen and the photosensitised production of singlet oxygen by PABA in the absence or presence of the two antioxidants.

1.1 STRUCTURE AND FUNCTION OF DNA

DNA is a molecule that occurs in all living cells. It exists as an unbound molecule in the cell cytoplasm in prokaryotes, e.g. *E. coli,* or as a protein-bound molecule in the nucleus of eukaryotes, e.g. human beings. It serves as the genetic material for living organisms. Its ability to transfer genetic information is attributed to its four main properties: precise replication, information storage, information transfer, and mutation. DNA consists of four nucleic acid bases (thymine, cytosine, guanine and adenine). The bases are the building blocks of the two polynucleotide strands that make up DNA. The two strands are long and antiparallel to one another, twisted about the same axis to form the DNA duplex (double helix) as shown in Figure 1.1 **[1]**. The bases are joined to a backbone of phosphate and deoxyribose units as shown in Figure 1.2 **[2]**.

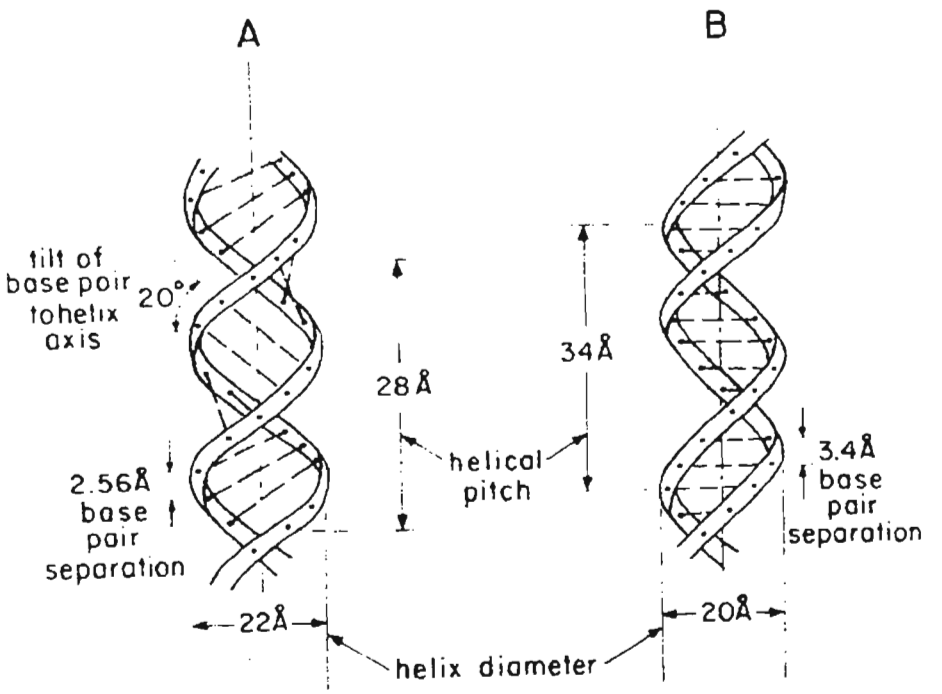

Figure 1.1: Dimensions of the two configurations of the DNA duplex. Configuration A and B are stable when the relative humidity is 40% and 66% respectively [1].

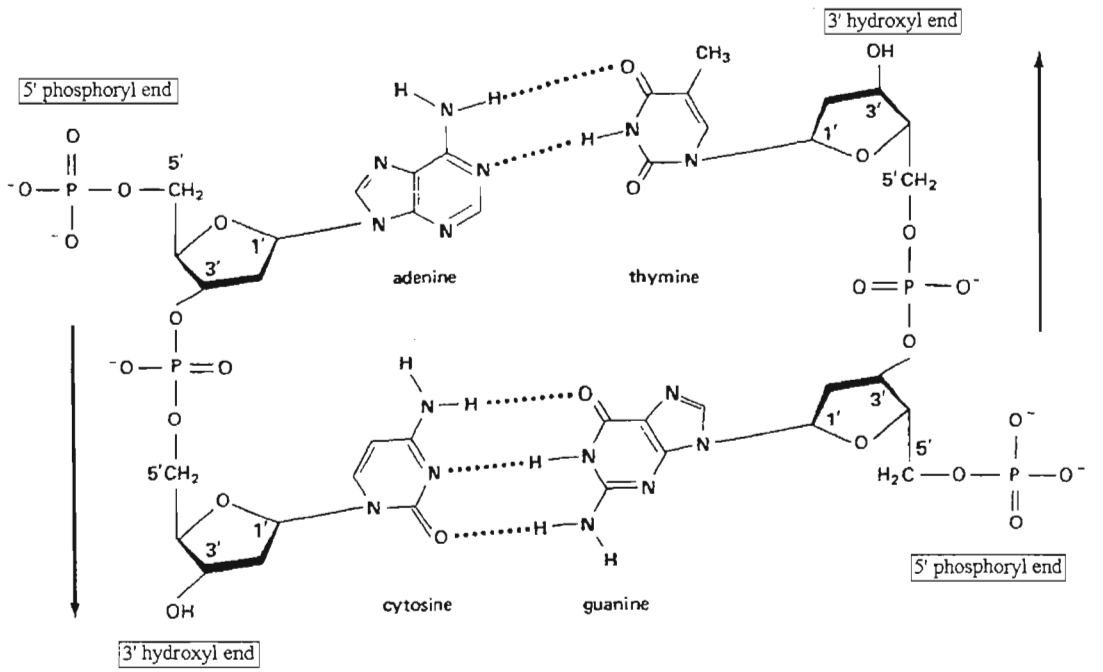

Figure 1.2: DNA duplex showing interstrand linkage between the bases, phosphate units and sugar units as well as the hydrogen bonds between the base pairs **[2]**.

There are three chemical bonds that make DNA very stable in a cell **[1]**. Firstly, the covalent bond that links atoms within nucleotide units. It does so via 3′,5′-phosphodiester bridges. The bridge extends from the 5′ carbon of one sugar unit to the 3′ carbon of the other sugar unit. This makes the chain very strong and resistant to breakage. Secondly, the antiparallel strands are held together by hydrogen bonds, which also maintain the molecular shape as seen in Figure 1.1. Thirdly, the hydrophobic interactions between the aromatic nitrogen bases that contribute substantially to molecular stability and to the exclusion of water molecules from the interior **[2]**. The genetic information is carried by a codon, the coding unit in DNA. In the codon, the sequence of the bases corresponds to the sequence of amino acids in a protein. The flow of information goes through an intermediate stage. DNA information is transcribed onto ribonucleic acid (RNA) by synthesis of a complementary RNA strand from the DNA template. This RNA genetic information is then used for protein synthesis. Any change in the identity of the codon leads to errors in the genetic information. This could ultimately lead to hereditary disorders and tumour cells.

1.2 STRUCTURAL CHANGES IN DNA

Mutations are reversible and rare changes in the genetic material that can arise in any cell. There are three criteria used to classify mutations. The effect on the phenotype, the molecular change on DNA, and the nature of the gene product **[2]**. Molecular change in DNA structure is considered as damage and has been linked to mutagenesis, carcinogenesis and cell death. This damage could be as a result of physical agents, chemical agents or a virus. Chemical mutagens act differently from one another on DNA. This could be a direct reaction with DNA to form new products or intercalating into the duplex and thus interfering with the genetic information. Examples of chemical mutagens are acridine, alkylating agents, base analogues and chemicals that react directly with DNA. Acridine removes or adds bases, whereas alkylating agents remove purines **[2]**. Base analogues occupy the sites of the bases of DNA thus giving false genetic information. A virus can also enter the host cell and cause similar damage to DNA.

1.3 PHYSICAL MUTAGENS AND PHOTOCHEMISTRY OF DNA

Physical mutagens, mainly radiation by gamma rays, X-rays, ultraviolet radiation (UV) or mechanical forces (physical stress), cause different types of damage to DNA [3]. Although the first physical mutagen to be studied was X-rays, there is more information on UV studies than on X-rays. This is because man is more likely to be exposed to UV radiation (200-400 nm) than to X-rays. In particular, the wavelength range 290-400 nm is of more concern since this is the region of the solar UV-spectrum that falls onto the earth's surface. Ultraviolet light of wavelength 254 nm has been used as a sterilising agent [2]. This and the correlation between the action spectrum for cell death and absorption of nucleic acids provides evidence that DNA is the principal cellular target for UV light [4]. DNA absorbs intensely at wavelengths between 254 nm and 260 nm, in the UVC region due to the presence of nucleic bases that serve as the chromophores for DNA. These bases possess excited states that can be populated on absorption of UV light. UVB is essentially absorbed in the epidermis and can cause mutation and initiate the formation of tumours [5]. UVA mainly causes tumour promotion and is more efficient in causing lipid peroxidation [5]. There is increasing evidence that shows that most, but not all, mutagens are carcinogens. The link between the physical mutagen (UV) and its carcinogenic effect has been widely investigated [6-8].

Ultraviolet radiation can be divided into three regions, namely UVA, UVB and UVC. These correspond to wavelengths 320 nm - 400 nm, 280 - 320 nm and 100 - 280 nm, respectively [9]. UVA and UVB wavelengths induce different types of cellular damage or lesions. The major products formed from DNA damage by wavelengths in the UVB region are the cyclobutane pyrimidine dimers and some pyrimidine-pyrimidone (6-4) photoadducts [10-15]. Pyrimidine dimers are known to produce deletion, frame shift and substitution mutations [16]. They have also been associated with base-to-base transitions in the genome. For instance, cytosine to thymine and cytosine-cytosine to thymine–thymine transitions are known [16]. Studies have suggested that an endogenous photosensitiser is responsible for dimer formation with radiation of wavelengths shorter than 365 nm since there is low dimer yield *in vivo* and

none in *vitro* [7]. For wavelengths longer than 365 nm, which fall in the UVA region, single-strand breaks and DNA-protein cross-links are common **[17]** as shown in Figure 1.3. There are many photoproducts of DNA known besides the dimers and adducts, but establishing their lethality and mutagenecity is a significant challenge.

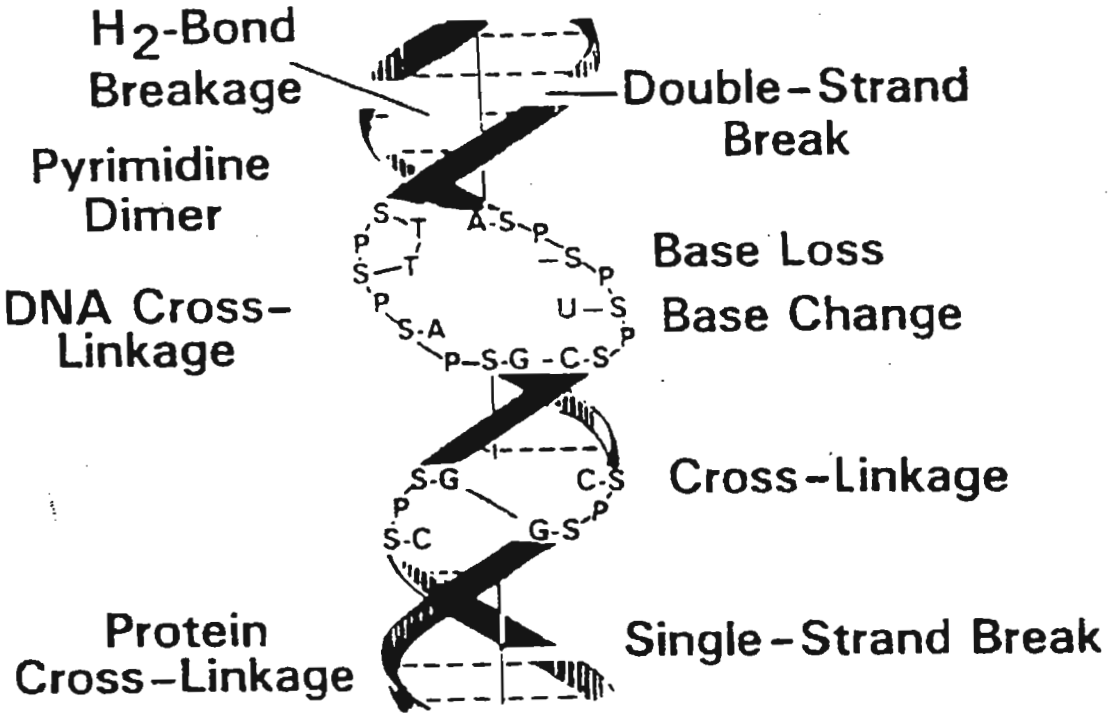

Figure 1.3: Strand break formation as a result of exposure to ionising gamma radiation [3].

1.4 THYMINE DIMERISATION IN DNA

Beukers and Berends **[18]** first isolated and identified *cis-syn* cyclobutane dimers from an irradiated frozen solution of thymine. The presence of the thymine dimer in DNA was confirmed by UV irradiation of DNA **[19]**. This confirmation was based on the similarities found between the thymine dimers formed in DNA and those obtained from frozen solution. Blackburn and Davies **[20]** provided direct evidence that the product of DNA irradiation was the *cis-syn* thymine dimer.

Nucleic acid bases are the chromophores responsible for the absorption of light energy in DNA. A base absorbs light energy and is promoted to an excited singlet state. This short-lived state rapidly relaxes by intersystem crossing to the first triplet excited state. Of the four DNA bases, thymine has the lowest energy for this triplet state **[21]**. The initial energy is localised in thymine resulting in a reaction with an adjacent base or by rapid energy transfer decays to ground state **[21]**. Because of the stereochemistry of DNA, the excited thymine is restricted to reacting with only a base in the same chain either above or below it. The major reaction involves [2+2] cyclo-addition of the 5-6 double bond of thymine on the 5′ side and either the 5-6 double bond or the C4 of the carbonyl on the 3′ side. This leads to a *cis-syn* cyclobutane dimer or an intermediate oxetane that is not thermally stable and hence decomposes to the 6-4 photoproducts (see Figure 1.4). The yield of *cis-syn* dimer is about 10 to 15 times that of the 6-4 photoproduct **[21, 22]**. Both the dimer and the photoadducts have been found to be significantly mutagenic **[23]**. The *cis-syn* thymine dimer isomer and (6-4) products are the main products. Nevertheless, small amounts of the *trans-syn* isomer are also formed on exposure of DNA to light of wavelengths greater than 290 nm, for example, sunlight. The *tran-syn* dimer results from the photodimerisation of two thymine molecules in which a thymine molecule is rotated through 180° about the nitrogen and carbon axis relative to the other as shown in Figure 1.4. Stable base stacking for pyrimidines in aqueous solution occurs in DNA **[24]**. In DNA, photodimerisation of adjacent thymine bases depends on the pattern of aggregation. The aggregation is most favourable in water. It is this interaction between bases in water that contributes to the stability of the double helix **[24]**. Gueron *et al.* **[25]** showed that stacked bases absorb UV light. In their findings, the first energy transfer occurs between bases in the dinucleotide, whereas the second energy transfer occurs at the excited singlet level. In this case the excitation is localised at the excimer. They also suggested a triplet excimer.

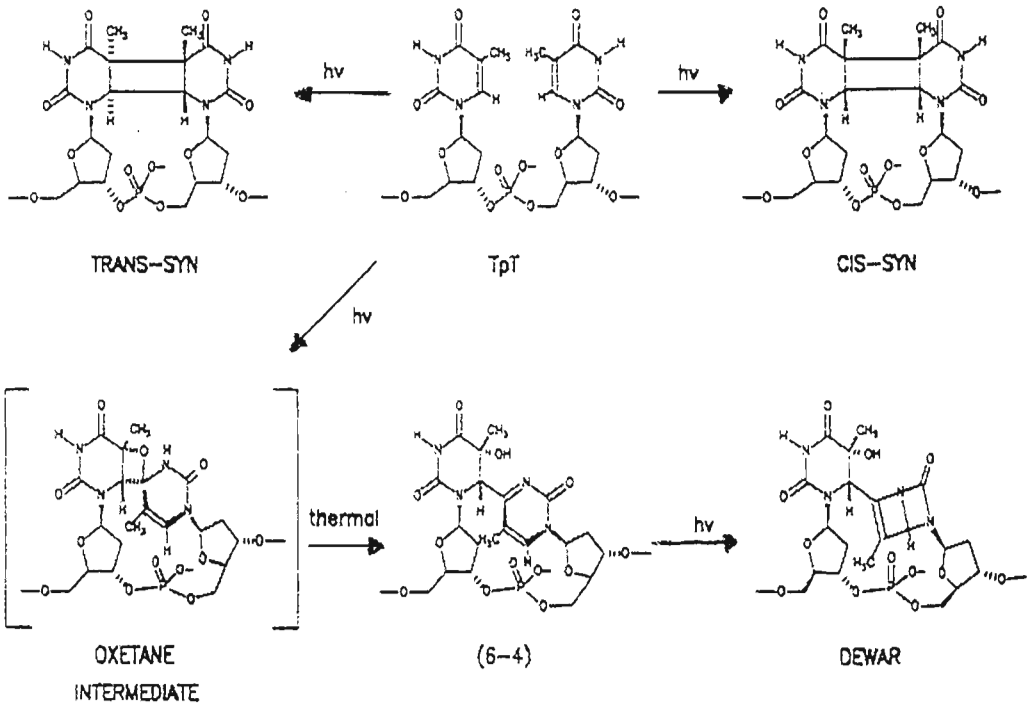

Figure 1.4: Reaction mechanism for the formation of *cis-syn* thymine dimer and other photoproducts in DNA **[21]**.

1.4.1 REPAIR MECHANISMS IN DNA

DNA repair is important in living cells since it determines the hereditary information that passes to the progenies. When UV-irradiated bacteria (mutants) are left in an incubator in the light compared to a similar set left in the dark, they contain fewer mutants **[2]**. This effect is called photoreactivation and is a result of DNA repair by some enzymes that depend on light called photoreactivating enzyme (PRE) **[8, 26-28]**. The most effective wavelength range for this reverse reaction is 310-400 nm. The enzyme acts on the photolesion by splitting the dimer into its monomers **[2, 23]**. This repair mechanism indicates that the dimers are the major cause of UV lethality. This is because the dimers are the only substrate for the enzyme and physiological change by the enzyme must be due to molecular change of the dimer to the monomer **[29]**.

DNA can also be repaired by excision that uses an enzyme in the dark as deduced from studies of repair mutants in *E. coli*. In this type of repair mechanism, the dimer and some adjacent base sequence are completely removed. The gap is resynthesized by DNA polymerase and sealed by the action of ligase. The strand opposite the gap determines the new sequence of the bases **[2, 26, 30]**. Figure 1.5 shows the excision repair mechanism of DNA.

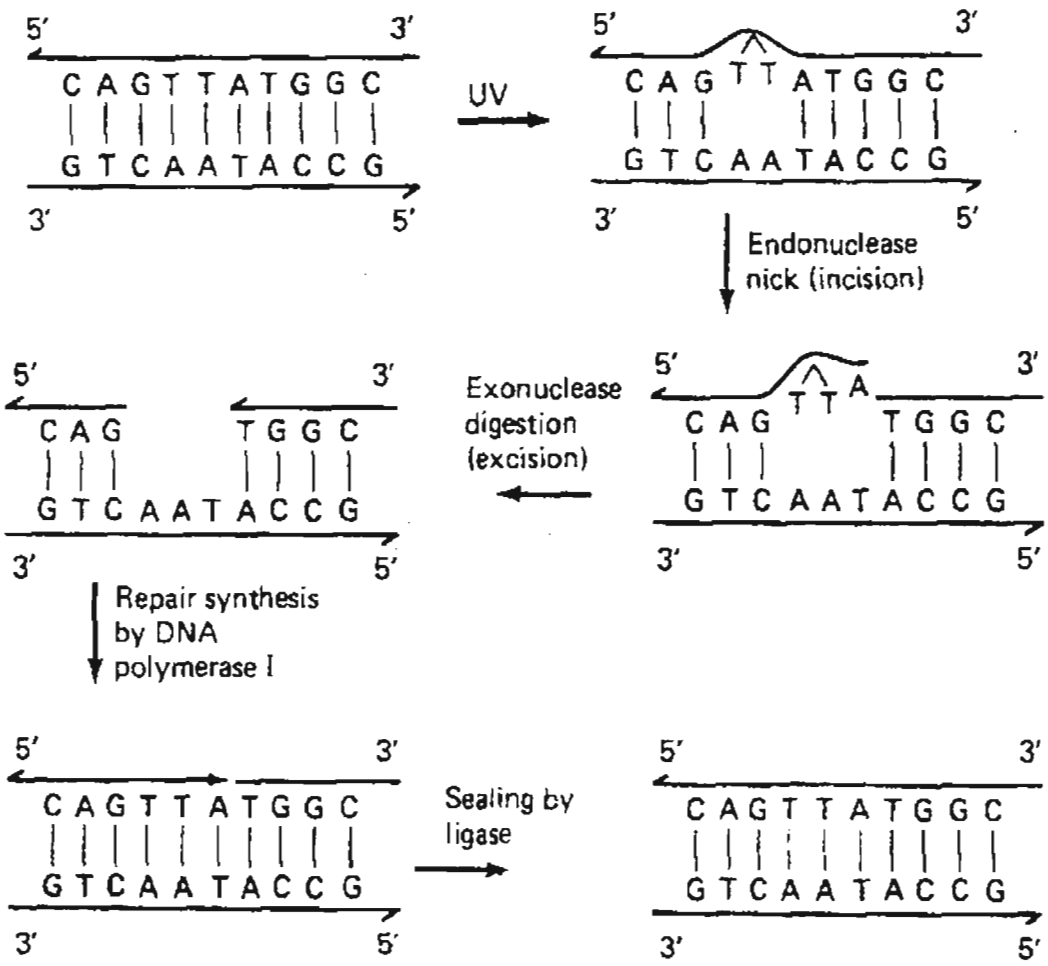

Figure 1.5: Excision repair of UV radiation damaged DNA **[2]**.

The two repair processes are, to a large extent, error free thus regenerating undamaged DNA. This is because there are almost no mutants at the end of these processes. *Xeroderma pigmentosum* (XP) is a rare hereditary disorder typical of defective repair of UV-induced damage to DNA. This disorder occurs mainly in the first step during the excision repair pathway owing to deficiency in repair enzymes **[2]**. Skin cancer incidence in people suffering

from this disease is 10^2 to 10^4 times greater than in non-sufferers. This suggests that thymine dimer is a major photolesion responsible for skin carcinogenesis **[2, 31, 32]**. There is a strong correlation between XP patients and their inability to repair (6-4) photolesions providing evidence for their mutagenicity **[33]**. There exists a third repair mechanism by enzymes studied in *E. coli*, namely postreplication repair, but the exact pathway has not been established. In this system, the dimers are neither split nor excised but are instead retained. Dimers lead to replication of daughter strands containing gaps at locations corresponding to the locations of dimers in the parental DNA. The presence of dimers causes distortion in base pairing between the daughter and parental strands during replication. Postreplication ensures that the gaps are filled by synthesis that is directed by polymerase **[2]**. The damage can overburden the repair enzymes leading to a situation where the enzyme cannot repair the damage and detrimental consequences arise.

1.5 PHOTODIMERISATION OF FREE THYMINE

In solution, thymine does not experience any stereochemical hindrance. Irradiation of free thymine in solution produces four isomers of the dimer **[21]** as seen in Figure 1.6. The total yield of the four thymine dimers is independent of temperature **[34]**, but is wavelength dependent with a quantum yield of formation of 0.1 at long wavelengths (265 nm – 270 nm) and a quantum yield for dissociation of 1.0 at short wavelengths (230 nm – 245) **[35, 36]**. Nevertheless, the type of photosensitiser, (and hence mechanism), temperature, concentration of monomer, pH of the solution, dielectric constant of the solvent, and irradiation wavelength determine the relative yield of the individual thymine dimer isomers **[34, 37, 38]**. Formation of dimers and photoadducts leads to loss of conjugation. The UV absorption spectrum of the monomer compared to that of the dimer indicates that this is the case. The monomer has an absorption maximum at 265 nm, which is not found in the dimer, and hence is a very useful property for distinguishing between the two. The UV absorption spectra for the monomer and dimer are shown in Figure 1.7. During irradiation of the dimer, an equilibrium exists between the monomer and the dimer. At 265 nm (the absorption maximum of the

pyrimidines) the dimer is favoured whereas at 245 nm, the monomer is favoured.

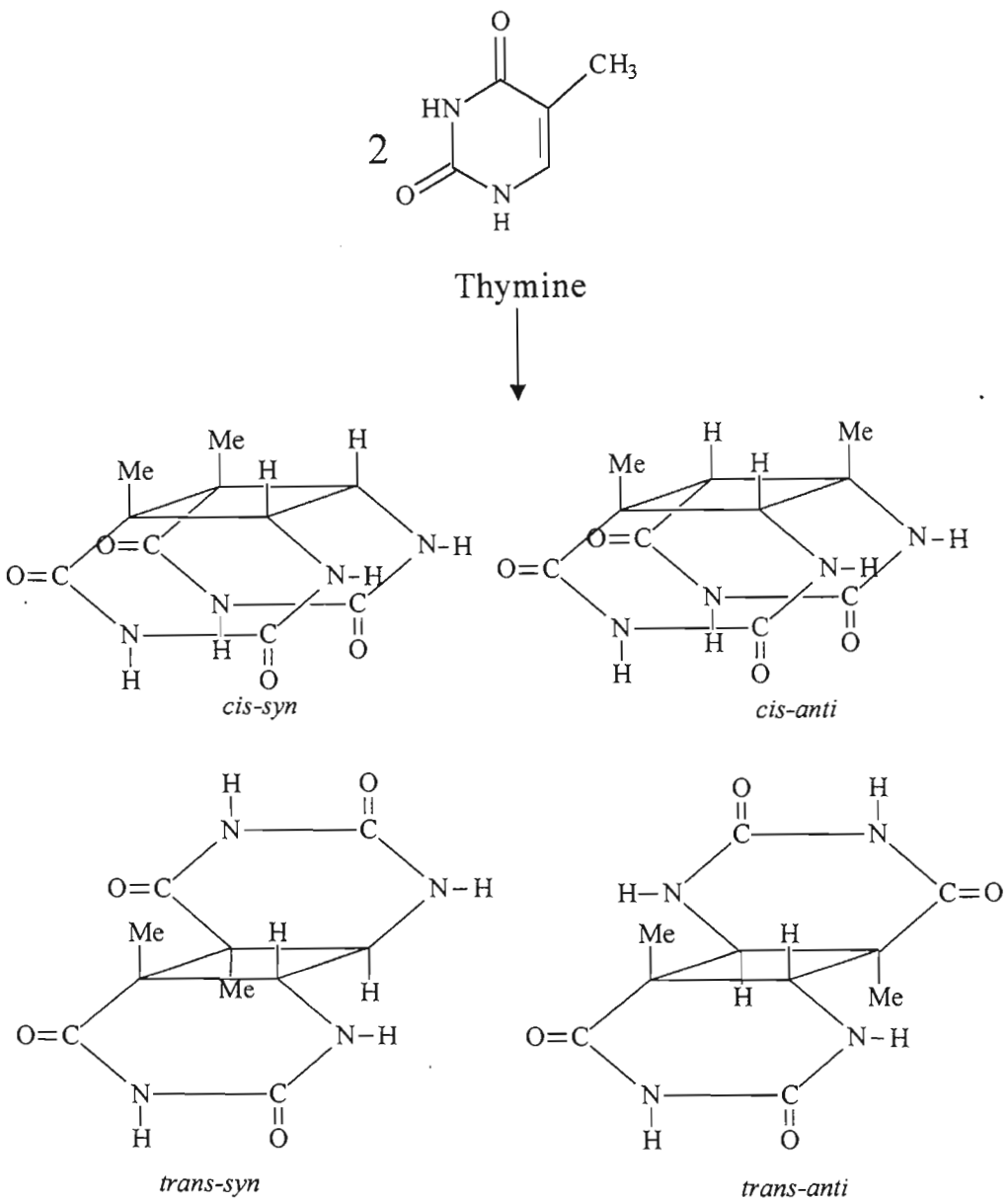

Figure 1.6: Four stereoisomers of thymine dimer formed on irradiation of an aqueous thymine solution **[39]**.

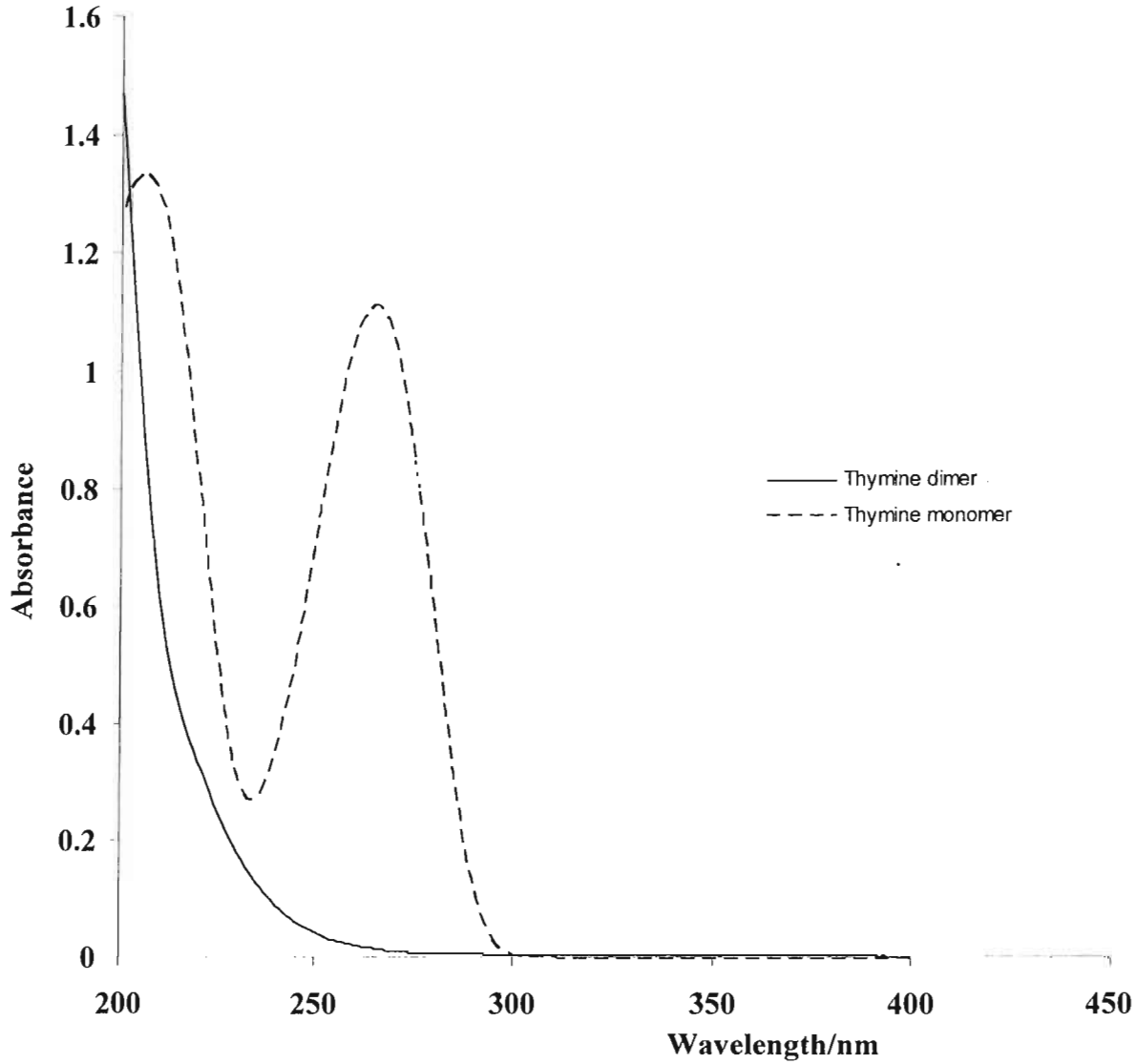

Figure 1.7: UV-absorption spectra of aqueous solutions of [thymine monomer] = 6.5 x 10^{-3} M and [*cis-syn* thymine dimer] = 1 x 10^{-3} M at pH 3.0 obtained in a 10 mm pathlength quartz cell with deionised water at pH 3.0 in the reference beam.

1.5.1 MECHANISM FOR PHOTODIMERISATION VIA THE TRIPLET STATE

The mechanism for photodimerisation of free pyrimidines in solution by direct UV irradiation ($\lambda < 290$ nm) is mainly via a triplet-state reaction **[40-43]**. This diffusion-controlled mechanism for dimerisation has received wide acceptance **[34, 40-43]**. A thymine molecule absorbs a photon and is excited to a short-lived singlet state that has a lifetime, τ, of 10^{-11} seconds **[39]**. This lifetime is too short to be involved in a dimerisation reaction of two thymine bases. Most of the singlet thymine, ^{1}T, formed undergoes radiationless transition to the ground state but a fraction undergoes intersystem crossing to give the long-lived triplet, ^{3}T, with a lifetime, τ, of 10^{-6} seconds, and a high intersystem crossing quantum yield **[39, 44]**. Triplet thymine may lose its energy by radiationless decay, oxygen bimolecular quenching and self-quenching. These processes compete with the process whereby ^{3}T collides with a ground state thymine to form a thymine dimer, T<>T. A schematic diagram shown in Figure 1.8 illustrates the processes explained. This phenomenon is mainly observed in dilute solutions where the concentration of thymine is less than 1×10^{-3} M **[34]**. At concentrations higher than 1×10^{-3} M, this mechanism accounts for only a fraction of the total dimer yield while most of the yield is via aggregate formation. The mechanism via aggregate formation is discussed in the next section.

1.5.2 MECHANISM FOR PHOTODIMERISATION VIA AGGREGATES

Many mechanisms have been proposed to explain the high dimer yield at high concentrations of thymine. One of these mechanisms is base stacking or commonly referred to as aggregation. For thymine concentrations above $1x\ 10^{-3}$ M, the high dimer yield has been attributed to the presence of stacked aggregates **[45]**. At thymine concentrations below 1×10^{-3} M, base stacking has been found to be negligible **[34]**. Foster **[46]** found that for thymine concentrations of less than 1.5×10^{-3} M, the yield is very low due to the lack of

aggregates. For higher concentrations, he found that the aggregate mechanism accounted for 75% of the total dimer yield. While studying the photoproduct distribution of cyclobutane dimer isomers, Morrison *et al.* **[47]** found that 80% of photoproducts in the case of *cis-syn* or *cis-anti* dimer formation involved stacked complexes whether in water or in organic solvents. Base stacking is more pronounced in aqueous solution where retention of the molecular structure of water is necessary **[34]**. Chan *et al.* **[48]** found that the high solubility of thymine in water increases the chances of base stacking.

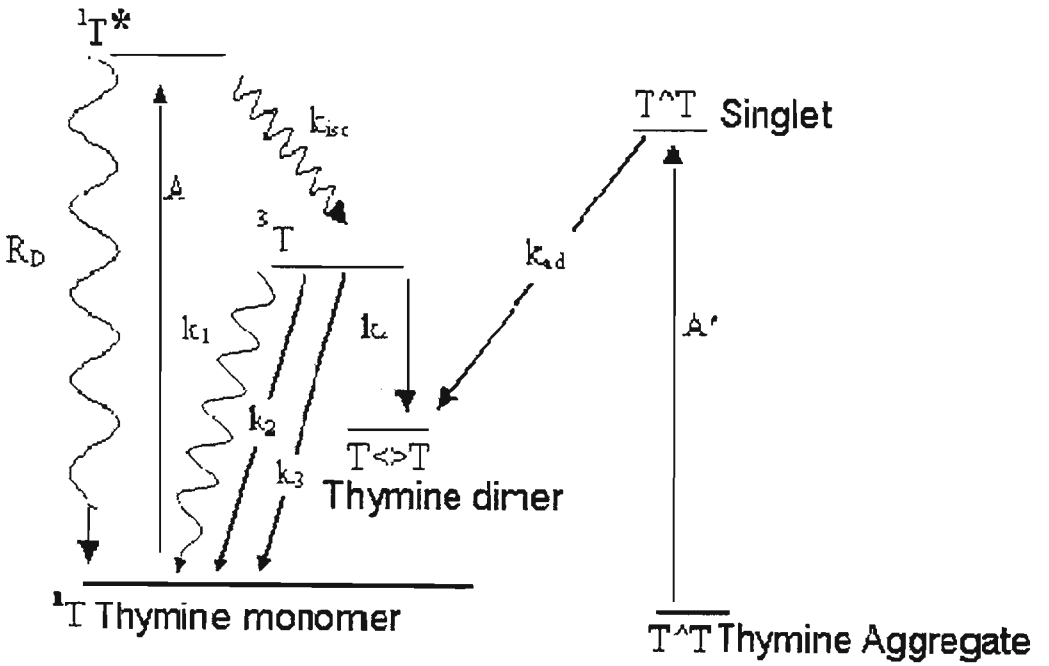

Figure 1.8: A Jablonski diagram showing the mechanism of thymine dimer formation via the triplet and aggregate mechanism.

Key to the diagram:

^1T	Thymine monomer	k_1	Radiationless decay of triplet thymine
$^1T^*$	Excited singlet thymine	k_2	Quenching by oxygen
$^3T^*$	Excited triplet thymine	k_3	Bimolecular self-quenching
T^T	Thymine aggregate	k_4	Dimerisation from triplet thymine
T<>T	Thymine dimer	k_{isc}	Intersystem crossing
A	Absorption of a photon by thymine monomer	k_{ad}	Dimerisation from excited singlet aggregate
A′	Absorption of a photon by thymine aggregate	R_D	Radiationless decay

Stacking in aqueous solution is mainly due to interaction of solvent molecules during the transition from thymine monomer to dimer **[49]**. The arrangement of water molecules around the stack is energetically favourable **[49]**. Goutam *et al.* **[50]** gave the energy for the stacking pattern of thymine as –32.22 kJ for every two moles of thymine. In their studies, they found that hydrogen bonds play a role in the development of the stacking pattern, i.e. whether vertical or planar. The hydrogen bonds were found to compensate in stacking patterns that are energetically unfavourable. The type of substituent on the solutes also has an effect on K_{st}, the equilibrium constant for stacking, with polar and alkyl groups increasing the value **[51]**. Van der Waal's forces also play a part in this mechanism **[34, 52-54]**. When these aggregates absorb photons there is interaction between sets of aggregate chromophores thus leading to formation of excimers. In aggregates, thymine dimerisation can proceed easily and directly from the singlet state. This is also shown in the Jablonski diagram in Figure 1.8.

Foster *et al.* **[46]** performed direct thymine photodimerisation experiments at 265 nm and did not use a photosensitiser; they proposed a dimerisation mechanism that took into account the formation and dissociation of aggregates. They found the rate constants for the formation and dissociation of the aggregates equal to 7.23×10^9 M^{-1} s^{-1} and 6.89×10^9 s^{-1}, respectively. The equilibrium for the formation of thymine dimer is concentration dependent. For dilute aqueous solutions of thymine of concentration range between 1×10^{-5} M and 1×10^{-4} M the dimer concentration is of the order of a few percent **[55]**. For irradiation at wavelengths greater than 280 nm without a photosensitiser, the equilibrium lies towards the formation of thymine dimer **[55]**. This equilibrium phenomenon was attributed to the close proximity of adjacent pyrimidine residues and their tendency to give stack conformation, which is favourable to cyclobutane dimer formation. The yield for *cis-syn* dimer is 43% and 33% via triplet and aggregate as precursors, respectively **[34]**. The quantum yield via aggregate is 0.014 at room temperature. The quantum yields for total dimer in these two cases are 0.00047 and 0.041 respectively. The latter value is independent of wavelength **[34]**.

1.6 SOLAR ENERGY AND THYMINE DIMERS

Figure 1.9 shows the relative spectral intensity and distribution of wavelengths of solar radiation that falls on the earth's surface.

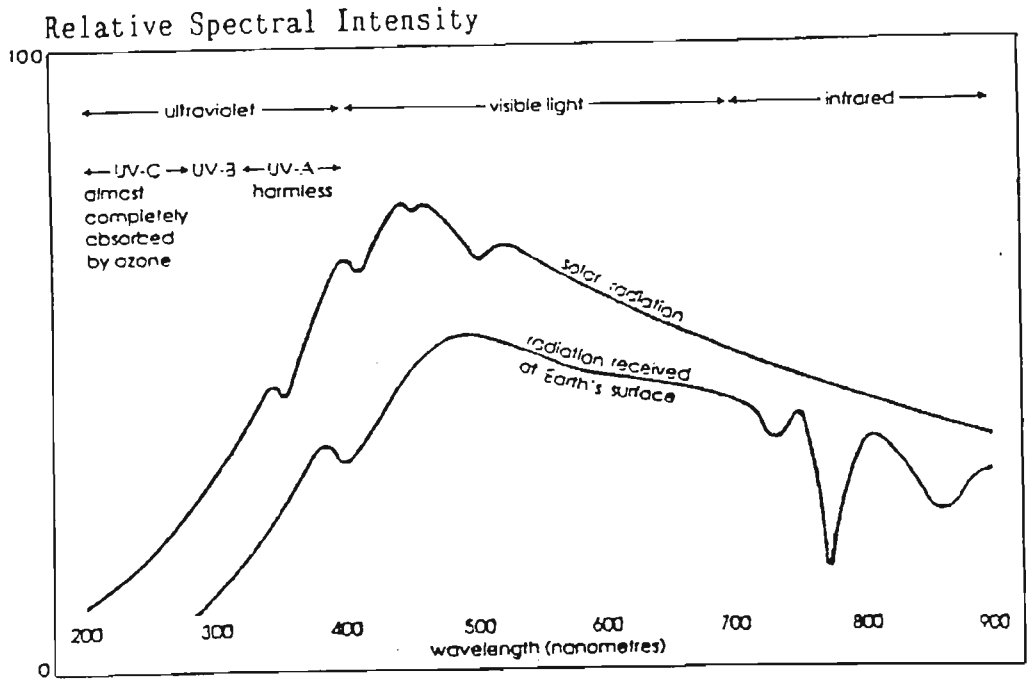

Figure 1.9: Relative spectral intensity and distribution of wavelengths of solar radiation falling on the earth's surface **[56]**.

The ozone layer filters off some of the solar UV-radiation and allows only UVA (320 - 400 nm) and UVB (290 - 320 nm) radiation to fall on the earth's surface. Solar UV radiation therefore does not favour direct dimerisation of thymine since thymine absorbs negligibly above 300 nm (See Figure 1.7). Depletion of the ozone layer increases the flux of radiation in the UVB region that penetrates the atmosphere and reaches the earth's surface. This could lead to direct dimerisation of thymine and consequently skin cancer. However, dimerisation via the thymine triplet state can be achieved by means of a photosensitiser. For this to occur, the singlet-excited state of the photosensitiser must be of a lower energy than that of singlet thymine whereas its triplet state must be of higher energy than that of thymine. This implies that light energy of

wavelengths greater 300 nm can be absorbed by the photosensitiser thus populating its singlet excited state (Figure 1.10 **[1]**). Depending on the quantum yield, all or a fraction of the singlets can undergo intersystem crossing to triplets. The thymine triplet may be populated by collision of ground state thymine and triplet sensitiser since the triplet of the sensitiser is of a higher energy than that of the ground state thymine **[57-60]**. There are many types of photosensitisers known including riboflavin, psoralen derivatives, acetone, acetophenone and benzophenone. The extent of photodimerisation depends on the efficiency (quantum yield) of intersystem crossing of the photosensitiser. If the quantum yield, φ_{isc}, is one, all singlets decay to triplets. If acetophenone is used as a photosensitiser of DNA bases, its triplet state is only able to populate the thymine triplet. The triplet states of the other bases cannot be populated by acetophenone. This implies that with acetophenone, only photosensitisation of thymine dimer is possible and not dimers of the other bases (see Figure 1.10). Nevertheless, acetone can populate the triplets of all the four bases in DNA thus giving many more photoproducts than acetophenone **[61]** (see Figure 1.10).

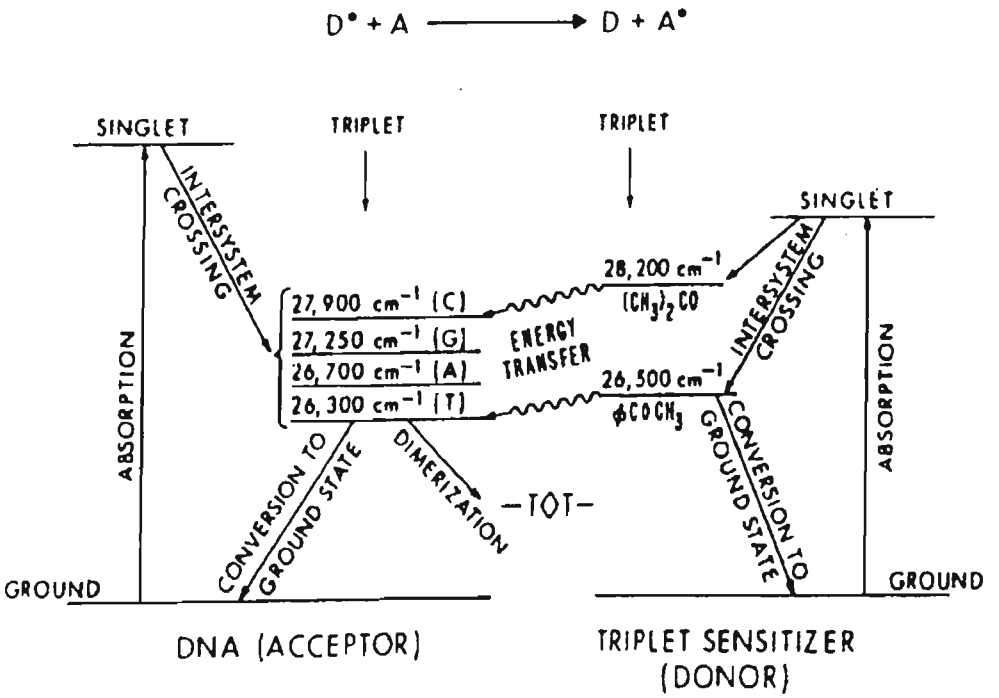

Figure 1.10: Jablonski diagram showing dimerisation of thymine by a triplet photosensitiser [1].

1.7 MECHANISM FOR PABA-PHOTOSENSITISED DIMERISATION

There is a lot of information on the photosensitising properties of PABA **[3, 4, 5]**. For thymine dimerisation to take place in the presence of a photosensitiser like PABA, the incident radiation must have a sufficiently long wavelength so that it is not absorbed by thymine but a short enough wavelength to be absorbed by PABA. On absorption of enough light energy, the photosensitiser is promoted to an excited singlet. Being a short-lived state, the singlet decays thus releasing its energy in different ways. These include internal conversion or fluorescence to the ground state. In water, fluorescence decay of PABA is very fast. It has a lifetime τ of 0.6 ns **[62]**. Brown and Revill **[63]** calculated it to be 0.79 ns. It can also decay to its triplet state by intersystem crossing and ultimately decay to the ground state. Figure 1.10 is a schematic Jablonski diagram in which the triplet sensitiser could be PABA. The efficiency of these decay processes is expressed in terms of the quantum yield. Data from fluorescence measurements on PABA **[63, 64]** and its low quantum yield of 0.07 for intersystem crossing **[65]** suggest a very low efficiency of photosensitised dimerisation. Sutherland **[66]** suggested that there was energy transfer from excited triplet PABA to DNA. This implies that the triplet state of the photosensitiser, PABA, has a lifetime long enough to photosensitise DNA. Nevertheless, it can decay to the ground state by phosphorescence or quenching. If the energy of the triplet photosensitiser is higher than that of ground state thymine, the energy is transferred to thymine thus resulting in triplet thymine. The efficiency of this process is dependent on the size of energy gap between the two triplets. The bigger the gap, the more efficient the transfer and the smaller the gap the less efficient the transfer. The transfer is basically diffusion controlled if the difference is between 1050 cm^{-1} and 1400 cm^{-1} **[67, 68]**. The energy difference between PABA and thymine is 300 cm^{-1} and hence the rate of energy transfer is expected to be of one or two orders of magnitude lower than the diffusion-controlled rate **[69, 70]**. Triplet thymine has a relatively long lifetime of approximately 10^{-6} s **[71]** and can decay by phosphorescence, quenching, or by colliding with a ground-state thymine to give a dimer.

1.7.1 PHOTOSENSITISED DIMERISATION IN AQUEOUS SOLUTION AT pH 3

In aqueous solution at 25 °C, a large fraction of PABA exists as a neutral molecule. Its protonation can thus occur in four different ways. It can be ionised, unionised or exist as a zwitterion. The following are the mechanisms for proton dissociation together with the associated pK values for PABA in aqueous solution **[72]**:

$^{+}H_3NC_6H_4COOH \rightarrow {}^{+}H_3NC_6H_4COOH^{-} + H^{+}$ pK = 3.49

$^{+}H_3NC_6H_4COOH \rightarrow H_2NC_6H_4COOH + H^{+}$ pK = 2.45

$^{+}H_3NC_6H_4COO^{-} \rightarrow H_2NC_6H_4COO^{-} + H^{+}$ pK = 3.79

$H_2NC_6H_4COOH \rightarrow H_2NC_6H_4COO^{-} + H^{+}$ pK = 4.83

$H_2NC_6H_4COOH \rightarrow {}^{+}H_3NC_6H_4COO^{-}$ pK = 1.04

At pH 3, it exists in the unionised form. At pH values closer to the pKs, both ionised and unionised forms exist. Its UV absorption and other physical properties are largely dependent on pH. For UV radiation of wavelength of 313 nm, at pH 6.88 its absorbance is 0.094 whereas at pH 3.97 the absorbance is 1.183 **[73]**. It absorbs maximum UV radiation at pH 3 **[65, 73]**.

Photosensitisers are molecules that can absorb a photon of light and are excited to their triplet states. The triplet state can then transfer the absorbed energy to another molecule. PABA is a molecule that can transfer the absorbed photon of light to another molecule. The form in which PABA exists affects its self-quenching efficiency although the process of photosensitisation in the two forms is essentially the same **[74]**. In unionised form, self quenching between excited triplet PABA and ground state PABA is very efficient with a rate constant of 5.0×10^{9} s^{-1}, whereas because of electrostatic repulsion in the ionised form, the rate of self-quenching is very low **[65, 74]**. PABA's photosensitising efficiency of free thymine base in aqueous solution was found to depend on pH and concentration of PABA **[65]**. By using a 10 mm thick Pyrex filter to allow light of wavelengths greater than 300 nm to impinge on the sample contained in a quartz cell of 1 mm pathlength, Rutherford *et al.* **[65]** suggested that at low PABA concentrations, there is competition for light

between PABA and thymine. Nevertheless, under our experimental conditions, thymine does not absorb light in this region. In solution, thymine can deprotonate to form a mono-anion, with a pK value of 9.9. Thus there will be minimal thymine ion concentration at low pH **[75]**. Paterson-Jones **[76]** and Malone **[77]** found that the deprotonation has an effect on the dimer yield. They found that dimer yield decreased with an increase in pH. This implies that at acidic pH there should be a relatively higher thymine dimer yield **[76]**. *Cis-syn* thymine dimer has pK_1 and pK_2 as 10.65 and 12.45, respectively **[35]**. Once formed in solution, it can revert to the monomer depending on the pH. At high pH, the cyclobutyl ring of the dimer opens up whereas in low pH it closes. This implies that it is most stable in lower pH **[78]**. In aqueous media, hydrogen bonding is possible for the unionised form of PABA although this is not likely to have a significant effect on the process of dimerisation **[74]**. Rutherford *et al.* **[65]** and Aliwell **[74]** proposed a mechanism for PABA-photosensitised dimerisation of thymine in aqueous solution at pH 3. From this mechanism the following kinetic rate equation for the formation of thymine dimer can be derived:

$$\frac{d[T <> T]}{dt} = k_1[P] + k_3[{}^1P^*] + k_6[{}^3P^*][T] + k_9[{}^3T^*][T] - \left\{k_2[{}^1P^*] + k_4[{}^3P^*] + k_5[{}^3P^*][P] + k_7[{}^3T^*] + k_8[{}^3T^*][T]\right\}$$

where P stands for ground state PABA, $^1P^*$ for excited singlet PABA, $^3P^*$ for excited triplet PABA, T for ground state thymine, $^3T^*$ for excited triplet thymine and T<>T for thymine dimer. The rate constants k_1, k_2, k_3, k_4, k_5, k_6, k_7, k_8 and k_9, represent singlet excitation of PABA, internal conversion, intersystem crossing, nonradiative decay of first excited singlet of PABA, self-quenching of PABA, diffusion-controlled energy transfer, nonradiative decay of the first excited singlet of thymine, self-quenching by thymine and thymine dimerisation respectively. The rate constants for the above proposed mechanism were obtained from the literature, by chemical actinometry and Stern-Volmer steady-state analysis as described below.

The value of k_1 was determined by chemical actinometry:

$$k_1 = \frac{I_a}{[P] \times N_a \times V} \qquad 1.1$$

where I_a is the rate of light absorbed in photons s^{-1}, [P] is the concentration of ground state PABA, N_a is Avogrado's constant and V is the volume of the solution irradiated. I_a was determined by chemical actinometry.

The values of k_4, k_5, and k_6 are limited by the ratios of k_4:k_6 and k_5:k_6 in the Stern-Volmer analysis. Rutherford *et al.* **[65]** determined these rate constants by plotting $I_a k_1 k_2/[\text{Dimer}]_{exp}$ against [PABA] as indicated in equation 1.4. The values of k_1 and k_2 were obtained from Equations 1.2. and 1.3 respectively **[79]**.

$$k_1 = \frac{k_3}{[k_2 + k_3]} = \varphi_{isc} = 0.07 \qquad 1.2$$

$$k_2 = \frac{k_9[T]}{(k_7 + k_8[T] + k_9[T])} = 0.05 \qquad 1.3$$

$$\frac{I_a k_1 k_2}{[\text{Dimer}]_{exp}} = 1 + \frac{k_4}{k_6[T]} + \frac{k_5[PABA]}{k_6[T]} \qquad 1.4$$

By using flash photolysis, Whillans and Johns **[80]** were able to determine the rate constant k_7, for the spontaneous nonradiative decay of triplet thymine, as 8 x 10^3 s^{-1}. They also found that the sum of rates for self-quenching and dimerisation (k_8 + k_9) to be 2.3 x 10^9 M^{-1} s^{-1}. They used the equation below to calculate k_8 and k_9

$$\varphi_{dimer} = \varphi_{isc} \times \varphi_{d,T} \quad 1.5$$

$$\varphi_{dimer} = \frac{k_9}{k_8 + k_9} \quad 1.6$$

where φ_{dimer}, $\varphi_{d,T}$ and φ_{isc} are the overall quantum yield of thymine dimerisation, the quantum yield from the triplet state of thymine, and the quantum yield of intersystem crossing of thymine, respectively. The values for Φ_{dimer} and Φ_{isc} were found to be 0.0008 and 0.0042, respectively. The values for k_8 and k_9 were found to be equal to 1.9×10^9 M^{-1} s^{-1} and 4.4×10^8 M^{-1} s^{-1}, respectively.

On the assumption that the self-quenching process is diffusion-controlled, the rate constant k_5 was taken as equal to k_d, the diffusion controlled rate of reaction. The maximum rate of diffusion of two similar neutral molecules was calculated by using the Stokes-Einstein approximation of the Debye-Smoluchowski equation **[81]**:

$$k_d = \frac{8RT}{3000\eta} \quad 1.7$$

where R = 8.314 J K^{-1} mol^{-1}, T = 298 K and $\eta = 8.937 \times 10^{-4}$ kg m^{-1} s^{-1} is the viscosity of water at 25 °C. Using these values in the equation, a maximum value of 7.4×10^9 M^{-1} s^{-1} was obtained for k_d. This is greater than the values 5.0×10^9 M^{-1} s^{-1} and 2.94×10^9 M^{-1} s^{-1} calculated by both Rutherford *et al.* **[65]** and Aliwell **[74]** respectively by simulation process for pH 3. This is not surprising since the repulsive electrostatic forces of diffusing ions would lower the rate of reaction. The intercept and gradient of equation 1.4 give $(1+k_4/k_6\,[T])$ and k_5/k_6 [T], respectively. By using the intercept, gradient and the calculated value of k_5, the values of k_4 and k_6 can be calculated.

The concentration of thymine used by Rutherford *et al.* **[65]** was constant at 1×10^{-2} M. Table 1.1 is a summary of the rate constants for the PABA-thymine aqueous system at pH 3 associated with the rate equation given earlier and used

to simulate the concentrations of thymine dimer obtained in the work of Rutherford *et al.* **[65]**.

Table 1.1: Rate constants for PABA-photosensitised thymine dimerisation in aqueous media at pH 3 obtained from the work of Rutherford *et al.* **[65]**.

k_1	I_a photons s^{-1}	k_6	$3.0 \times 10^7\ M^{-1}\ s^{-1}$
k_2	$1.0 \times 10^{11}\ s^{-1}$	k_7	$8.0 \times 10^3\ M^{-1}\ s^{-1}$
k_3	$1.6 \times 10^9\ s^{-1}$	k_8	$2.0 \times 10^9\ M^{-1}\ s^{-1}$
k_4	$8.0 \times 10^6\ s^{-1}$	k_9	$5.0 \times 10^8\ M^{-1}\ s^{-1}$
k_5	$5.0 \times 10^9\ M^{-1}\ s^{-1}$		

The second rate constant, k_2, in the work of Rutherford *et al.* **[65]** represents internal conversion. This part of the mechanism was considered as two steps in the mechanism proposed by Aliwell **[74]**, i.e. fluorescence and nonradiative decay represented by k_2 and k_3. The steps represented by k_3, k_4, k_5, k_6, k_7, k_8 and k_9 in Rutherford's work are the same as those in Aliwell's work represented by k_4, k_5, k_6, k_8, k_{10}, k_9 and k_{11}, respectively. Aliwell omitted the value for k_7 representing quenching of the triplet photosensitiser by thymine. It was considered negligible in the presence of a PABA concentration of 1 mM.

Table 1.2 is a summary of the rate constants obtained from the work of Aliwell *et al.* **[74]** for the PABA-photosensitised dimerisation of thymine in aqueous solution at pH 3. These rate constants were used to simulate the concentrations of thymine dimer obtained experimentally.

Table 1.2: Rate constants for PABA-photosensitised thymine dimerisation in aqueous media at pH 3 obtained from the work of Aliwell *et al.* **[74]**.

k_1	$6.306 \times 10^{-3}\ s^{-1}$	k_6	$7.40 \times 10^9\ M^{-1}\ s^{-1}$
k_2	$1.27 \times 10^9\ s^{-1}$	k_8	$1.339 \times 10^8\ M^{-1}\ s^{-1}$
k_3	1.49×10^{10}	k_9	$1.9 \times 10^9\ M^{-1}\ s^{-1}$
k_4	$1.22 \times 10^9\ s^{-1}$	k_{10}	$8 \times 10^3\ s^{-1}$
k_5	$7.20 \times 10^6\ s^{-1}$	k_{11}	$4.4 \times 10^8\ M^{-1}\ s^{-1}$

The mechanisms proposed by both Aliwell **[74]** and Rutherford *et al.* **[65]** for PABA-photosensitised thymine dimerisation at pH 3 are similar and satisfactory in that they were able to account for the observed yield of thymine dimer. Their mechanisms, however, did not account for base stacking.

Kilfoil and Salter **[45, 79]** observed that at high thymine concentrations, dimer yield was not dependant on the monomer concentration. To reconcile this discrepancy they suggested a combination of triplet and base stacking mechanisms. While using acetophenone and benzophenone as photosensitisers, they proposed a mechanism where a triplet photosensitiser populates triplet aggregates by energy transfer. The triplet aggregate then decays to give a dimer or two molecules of the monomer. The mechanism they proposed is as follows: At high thymine concentrations, the number of monomer molecules is greater

$$T + T \rightleftharpoons T^{\frown}T \qquad k_1 = 1 \times 10^9\ M^{-1}s^{-1}$$

$$T^{\frown}T + {}^3P \longrightarrow {}^3T^{\frown}T + P \qquad k_2 = 2 \times 10^9\ M^{-1}s^{-1}$$

$${}^3T^{\frown}T \longrightarrow T <> T \qquad k_3 = 1 \times 10^9\ M^{-1}s^{-1}$$

$${}^3T^{\frown}T \longrightarrow 2T \qquad k_4 = 3 \times 10^8\ M^{-1}s^{-1}$$

than the aggregates **[51, 53, 82]**. Therefore, the monomer could compete more efficiently for a photosensitiser assuming a diffusion-controlled reaction. In their model, for efficient dimerisation from aggregates to take place, all the aggregates have to associate with the photosensitiser. This would result in a great reduction in surface area of the aggregate. This reduction in the surface area is important in hydrophobic interactions **[83]**. To maximise dimer yield, they further proposed the two mechanisms below. The first mechanism accounted for the association between the aggregate and the photosensitiser with triplet energy transfer efficiency of unity. The second mechanism represented base stacking between thymine and photosensitiser.

$$T^{\frown}T + P \longrightarrow T^{\frown}T^{\frown}P$$

$$T^{\frown}T^{\frown}P \xrightarrow{h\nu} {}^{1}T^{\frown}T^{\frown}P$$

$${}^{1}T^{\frown}T^{\frown}P \longrightarrow {}^{3}T^{\frown}T^{\frown}P$$

$${}^{3}T^{\frown}T^{\frown}P \longrightarrow T <> T + P$$

$${}^{3}T^{\frown}T^{\frown}P \longrightarrow 2T + P$$

and

$$P + T \longrightarrow P^{\frown}T$$

$$P^{\frown}T \xrightarrow{h\nu} {}^{3}P^{\frown}T$$

$${}^{3}P^{\frown}T + T \longrightarrow T <> T + P$$

1.8 SINGLET OXYGEN, UV RADIATION, AND DNA DAMAGE

Oxidation is very important in the body. Oxidative metabolism is necessary for cell survival. Most of the oxygen in the cell is used for this purpose. Unfortunately, oxidation produces side effects that are harmful to the body. Free radicals and reactive oxygen species (ROS) are good examples **[84]**. The production and presence of ROS is referred to as oxidative stress. Radiation in the UVA region causes more oxidative stress than radiation from the UVB region. Literature reports **[5]** indicate that UVA is 10 times more efficient than UVB in causing lipid peroxidation (a form oxidative stress).

The ROS of interest in this work was singlet oxygen, the lowest excited state of molecular oxygen, since it can react with nucleic acids and therefore damage biological systems. The role of singlet oxygen in carcinogenesis and cytotoxicity of the cell is well documented **[85]**. It is a major reactive species produced during photosensitisation by UV radiation **[86]**. In recent years it has attracted considerable attention because of its involvement in skin photosensitivity and cancer. Since the discovery that light in the presence of oxygen and sensitizing dyes kills microorganisms, photosensitised oxidation has become of great interest to chemists and biologists. This pathological effect

is called photodynamic action. It includes cell damage, induction of mutations or cancer and cell death **[87]**.

1.8.1 ELECTRONIC STRUCTURE OF OXYGEN

The most stable form of oxygen, molecular oxygen, has two unpaired electrons in its ground state. Molecular orbital theory gives it the following electronic configuration and spin configuration:

$(1\sigma_g)^2(1\sigma_u)^2(2\sigma_g)^2(2\sigma_u)^2(3\sigma_g)^2(1\pi_u)^4(1\pi_g)^2$ electronic configuration

$(\sigma_{1s})^2(\sigma_{1s}^*)^2(\sigma_{2s})^2(\sigma_{2s}^*)^2(\sigma_{2p}x)^2(\pi_{2p}y)^4(\pi_{2p}y^*)^2(\sigma_{2p}x^*)^0$ spin configuration

This configuration is represented by $^3\Sigma_g^-$ in Figure 1.11. The electronic properties of oxygen are determined by the six π molecular orbitals. Unpaired electrons in stable molecules are rare, hence the electronic configuration of oxygen makes it a very reactive species. Two paired electrons occupy the same π_g molecular orbital in one of the excited states. This configuration is represented by $^1\Delta_g$ (0.98 eV) in Figure 1.11. It is the lower energy form of singlet oxygen, $^1O_2$ with a lifetime of 2×10^{-6} seconds in water **[88, 89]** that is reactive and is what is commonly referred to as "singlet oxygen". The higher energy species is represented as $^1\Sigma_g^+$ (1.63 eV) and the electrons occupy two different π_g molecular orbitals but with opposite spin **[90]**. It has a lifetime of approximately 10^{-11} seconds in water **[91]**. This species rapidly converts to the lower energy and long-lived form **[90, 92]**. Nevertheless, the short-lived singlet state can react with materials that are in relatively high concentration **[93]**. Electronic transitions from the ground state to any one of these species is forbidden but is observed in absorption and emission in the upper atmosphere with zero-zero transitions at 1269 nm and 762 nm **[94]**.

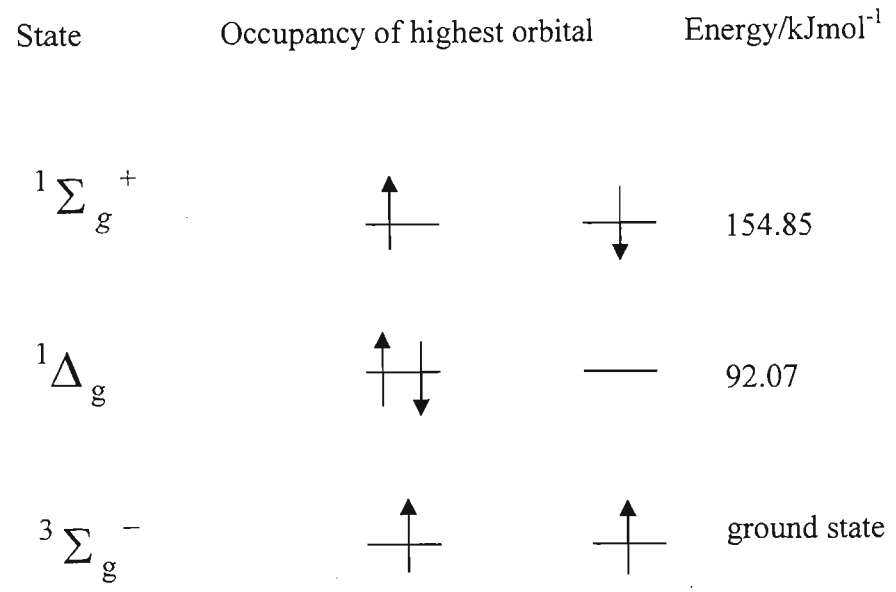

Figure 1.11: Energy levels for the three electronic structures of oxygen.

1.8.2 PHOTOSENSITISED FORMATION OF SINGLET OXYGEN

Singlet oxygen can be formed by any one of the following methods: via a photosensitiser (a light-absorbing substance), by gaseous discharge in which an electric current is passed through a gas or vapour, by an exothermic reaction in which singlet oxygen is generated as a product and by enzyme-catalysed reactions. These last two mentioned reactions can be chemiluminescent because of the radiative decay of singlet oxygen. Photosensitisation, a method that frequently occurs in nature **[95]** is the method used to generate singlet oxygen in this work and is therefore discussed here.

The first step in the process of photosensitization is the absorption of a photon of radiation by a photosensitiser to produce the first excited state of the photosensitiser ($^1P^*$) which can by intersystem crossing go to the excited triplet state ($^3P^*$) as shown in Equations 1 and 2 overleaf **[96]**. In the presence of oxygen, there are two competing processes referred to as Type I and Type II **[97]**. In Type I, the excited photosensitiser can react with either substrate or the solvent. This reaction is either by hydrogen abstraction or electron transfer to give a radical or a radical ion **[97]**. The radical ion formed can react with

oxygen to give superoxide radical anion **[98]**. At low pH the superoxide radical anion can protonate to give the reactive $HO_2^{\bullet}$ radical. In Type II, the excited triplet photosensitiser can react with ground-state oxygen to produce mainly singlet oxygen by energy transfer. Nevertheless, electron transfer can also occur in this Type to give an oxidized product of the photosensitiser and superoxide radical anion. Figure 1.12 is a summary of the mechanisms involved in both Type I and Type II processes.

$$P + h\nu \longrightarrow {}^1P^* \qquad 1$$

$${}^1P^* \longrightarrow {}^3P^* \quad \text{intersystem crossing} \qquad 2$$

$${}^3P^* + {}^3O_2 \longrightarrow P + {}^3O_2{}^* \quad \text{energy transfer} \qquad 3$$

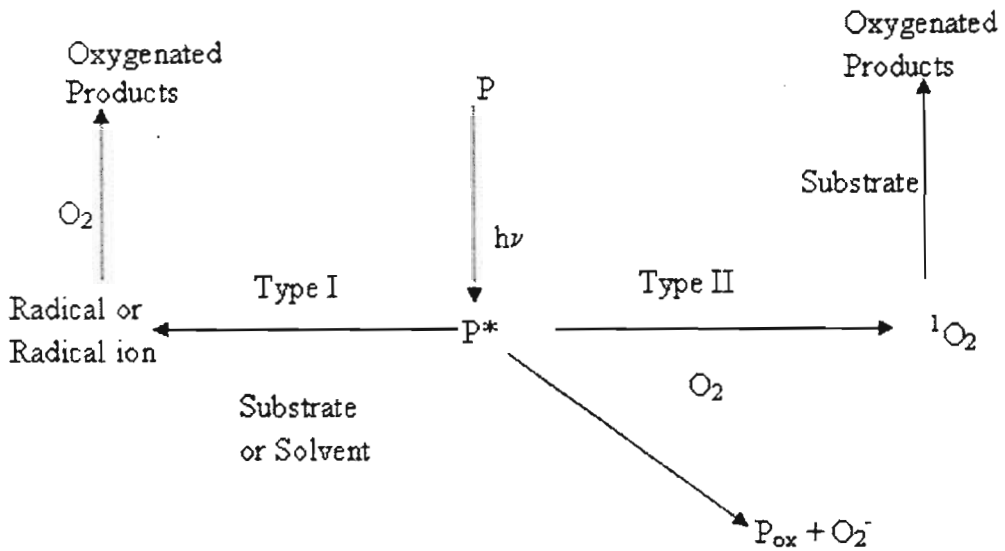

Figure 1.12: A summary of Type I and Type II processes for the reaction of a photosensitiser **[97]**.

Many organic compounds can act as photosensitisers and thus are able to produce singlet oxygen. The energy required to promote ground-state molecular oxygen to the excited singlet is 95 kJ mol^{-1}. The photosensitiser used in this work was PABA. Its triplet excited state energy has been reported as 313.9 kJ mol^{-1}, which clearly indicates that it has enough energy to promote ground state molecular oxygen to its first excited singlet state **[99]**.

1.8.3 *IN VIVO* PHOTOSENSITISATION OF SINGLET OXYGEN

When irradiated with UVB radiation, thymine in DNA has been shown to photosensitise the formation of singlet oxygen **[100]**. Triplet excited thymine can react with molecular oxygen by either energy transfer to produce singlet oxygen or by electron transfer according to the mechanism below **[101]**:

$$^3T + O_2 \longrightarrow T^{\cdot +} + O_2^{\cdot -}$$
$$^3T + O_2 \longrightarrow T + {}^1O_2\ (^1\Delta_g)$$

where T stands for thymine. The second reaction is feasible because the triplet states of the DNA bases have sufficient energy, usually greater than 300 kJ mol^{-1}, to transfer their excitation energy to molecular oxygen **[102]**. The singlet oxygen quantum yield Φ_Δ is related to the triplet quantum yield, Φ_T, of a DNA base, like thymine, by Equation 1.8 shown below:

$$\Phi_\Delta = \Phi_T S_\Delta \qquad 1.8$$

where S_Δ is the fraction of triplet state quenched by singlet oxygen. The value of Φ_Δ for thymine is reported as approximately 0.07 in acetonitrile and that of Φ_T is 0.006 in water **[101]**. These values for thymine indicate that photosensitisation of singlet oxygen by thymine is possible. Indeed Bishop *et al.* **[101]** have reported thymine photosensitisation of singlet oxygen by using UVB irradiation. In 75% dimethylsulfoxide (DMSO) and 25% water, Zhang *et al.* **[103]** calculated the rate constant for singlet oxygen reacting with thymine as 2.5×10^{-4} M^{-1} s^{-1}. This implies that thymine has conflicting roles in the presence of singlet oxygen. It can either be a photosensitiser or a sink for singlet oxygen.

Other examples of *in vivo* photosensitisers that produce singlet oxygen are flavin mononucleotide, flavin adenine dinucleotide, retinal and proteins **[104]**. Some photosensitised reactions can be used for treatment of skin diseases such as *psoriasis*. The treatment of this disease involves a combination of psoralens and UVA radiation known as PUVA therapy; although its use is controversial

because it may enhance skin cancer [104]. Some diseases can lead to excessive formation of singlet oxygen, a good example are the *porphyrias* [104]. Some drugs like tetracycline antibiotics have been shown to sensitise the formation of singlet oxygen [105].

1.8.4 DAMAGE CAUSED BY SINGLET OXYGEN

Singlet oxygen, $^1\Delta_gO_2$, has a short lifetime in water compared with organic solvents [88, 89]. It is capable of penetrating cells and causing damage to DNA resulting in single-strand breaks and base alteration [105-107]. Possible involvement of $^1\Sigma_g^+$ O_2 in photooxidation has also been suggested [108]. Genetic changes induced by singlet oxygen have been reported in photodynamic inactivation of yeast cells photosensitised by acridine orange [109]. It can undergo oxidative reactions with a number of cell constituents. For example, it can alter amino acids, nucleic acids and proteins by oxidative damage thus resulting in the formation of antigens, which manifest in allergic responses [110]. It is known that exposure of skin to UVB radiation can result in photocarcinogenesis and that the transformation of skin cells to neoplastic growth involves endogenously photosensitised formation of $^1O_2$ [111]. The relative importance of the photodamage depends on the type of photosensitiser, the efficiency of energy transfer and the concentration of oxygen [104].

1.8.5 QUENCHING OF SINGLET OXYGEN

Singlet oxygen quenching can involve a route where the quencher (Q) undergoes either no ultimate chemical change (physical quenching), or a chemical reaction resulting in new products [112]. Both the photosensitser (P) and the quencher can quench and react with singlet oxygen in some cases. The sequence of reactions below summarizes the quenching process in which singlet oxygen is involved in the presence of a photosensitiser. Different photoproducts are formed depending on the nature of the photosensitiser (P). PO_2 represents the organic peroxide oxidation product of a photosensitiser.

$$P + h\nu \longrightarrow {}^1P^*$$

$${}^1P^* \longrightarrow {}^3P^*$$

$${}^3P^* + {}^3O_2 \longrightarrow P + {}^1O_2^*$$

$${}^3P^* + Q \longrightarrow P + Q$$

$$P + {}^1O_2^* \longrightarrow PO_2 \quad \text{chemical quenching}$$

$$Q + {}^1O_2^* \longrightarrow \text{physical quenching}$$

$${}^1O_2^* \longrightarrow {}^3O_2 + h\nu \quad (h\nu = 1270\,\text{nm} - \text{luminescence emission})$$

Our investigation involved PABA as a photosensitiser, and the presence or absence of oxygen in the reaction media besides the antioxidants and thymine. From the sequence of reactions above, it is evident that PABA and singlet oxygen affect the effective concentration of one another. The presence or absence of oxygen in the solution has a pronounced effect on the photoproducts formed. It has been reported that photo-adducts of thymine-PABA are considerably reduced in the presence of oxygen. The possible reasons for this are firstly that oxygen quenches triplet PABA. Secondly, oxygen is involved in electron or radical scavenging. In this way it affects the concentrations of important intermediate species in the reaction mechanisms **[113]**.

1.9 PARA-AMINOBENZOIC ACID

Para-aminobenzoic acid shown in Figure 1.13, once considered as vitamin K, was until very recently used in sunscreen formulation as an active ingredient for photoprotection against UVB radiation **[114, 115]**. It has a λ_{max} of 288 nm in water and does not absorb wavelengths greater than 320 nm **[116]**. Earlier researchers have found that it could scavenge reactive oxygen species like singlet oxygen and protect DNA against UV and free radical damage, in addition to having mild anti-inflammatory activity **[117, 118]**. It was a widely used ingredient because it enjoyed the advantage of having the ability to penetrate the horny layer of the skin within a short period. Nevertheless, it had some disadvantages like stinging sensitisation, drying the skin and staining clothes **[119]**. More recent studies have implicated it and its derivatives as potential photosensitisers **[120]**. There has been a decline in the use of PABA in sunscreen formulations because of reports on its photoallergy effects it

causes on the skin. Studies have shown that *E. coli* cells are killed when exposed to radiation of wavelength of 313 nm in the presence of PABA **[120-123]**. Once again photoreactivation studies have implicated pyrimidine dimers as the major photolesion caused by PABA. This was confirmed by Sutherland *et al.* **[66]** when they showed that PABA can penetrate human cells and cause photosensitised dimerisation of pyrimidine dimers in DNA. Their studies and those of many others have confirmed that PABA can photosensitise dimerisation of thymine in DNA **[124]**. Two conflicting properties of PABA, protection and photosensitisation, are concentration dependent. For example, irradiation of *E. coli* with radiation of wavelength of 313 nm at high PABA concentrations, such as solutions containing 0.1% of PABA, causes sensitisation almost 8.5 times more than when the concentration is 0.001% **[125]**. Protection by PABA is also pH-dependent. At lower pH, it exists as a zwitterion and its overall absorbance increases thereby providing high protection at low concentration [**73**].

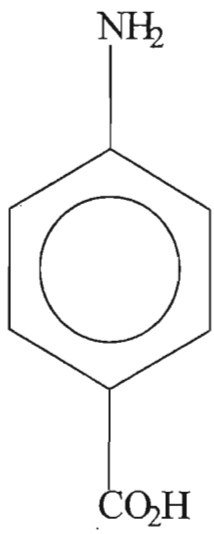

Figure 1.13: The structure of *para*-aminobenzoic. acid.

Besides thymine dimers, there are a number of other photoproducts that are formed upon irradiating PABA with UV radiation either in the presence or absence of other chemicals **[43, 113]**. The types and number of photoproducts formed by PABA-photosensitised reactions are indicative of its potential as a mutagen. Some of the major photoproducts are 4-amino-3-hydrobenzoic acid,

4-aminophenol and 4-[(4'-hydroxyphenyl)amino] benzoic acid **[85, 113]**. Allen *et al.* **[120, 126, 127]** showed that although PABA can quench singlet oxygen with a very high rate constant, it could also photochemically produces singlet molecular oxygen when irradiated under constant sunlight conditions and hence produce a steady-state concentration of singlet oxygen. Thus singlet oxygen could easily be implicated in the dermatological effects, like photoallergy and skin irritation, that are associated with PABA **[128]**.

1.10 PHOTOPROTECTION AND SUNSCREENS

Besides causing skin cancer, UV radiation incident on the earth's surface is also associated with erythema, i.e. reddening of the skin. Stratospheric ozone depletion and change in lifestyle have continually made man more vulnerable to these harmful effects of UV radiation. Sunscreens have been developed and are used for protection against the deleterious effects of UV radiation. The ability of a sunscreen to protect the skin from the rays of the sun is measured in terms of a conventionally accepted photoprotective index called the Sun Protection Factor, SPF. It is the ratio of the UV energy required to produce a minimal erythymal dose (MED) on protected skin to the UV energy required to produce an MED on unprotected skin **[129, 130]**:

$$\text{SPF} = \frac{\text{Minimal Erythymal Dose in sunscreen - protected skin}}{\text{Minimal Erythymal Dose in non - sunscreen - protected skin}}$$

This factor is normally measured and tested at a sunscreen application density of 2 mg cm^{-2} of the skin but studies have shown that application by consumers is typically 0.5 mg cm^{-2} **[5]**. This, coupled with the fact that DNA damage is measured in terms of thymine dimer formation, renders the protection by sunscreens unreliable. Some of the ingredients have been shown to be harmful when absorbed by the skin. No sunscreen provides total protection from photodamage **[5]**.

There are many active ingredients found in sunscreens formulations. They are contained in sunscreens so as to reduce the harmful effects that results from

exposure to UV radiation. The ingredients in a typical sunscreen formulation are given in Table 1.3. Other than the absorbers that provide protection, the other ingredients of sunscreens are vehicles that make them more appealing and easily applied on the skin. In choosing the ingredients for a sunscreen formulation the following requirements must be put into consideration **[131]**.

These requirements are divided into three categories namely, physical requirement, toxicological requirements and formulation requirements. The first category requires that the ingredients be good absorbers in the UVB and UVA regions, chemically stable, non-staining and lastly exhibiting minimal absorption through the skin and should not absorb water. The second category requires that the ingredient be non-irritating, non-sensitising and non-toxic. The last category requires that the ingredient be soluble in cosmetics, easy to handle and formulate and be stable in cosmetic bases.

Table 1.3: Typical ingredients found in sunscreens **[132]**.

Substance	**Percentage**
UV absorber	3-15
Polyethylene	2-15
Water	40-65
Softener	15-30
Emulsifier	3-10
Preservative and antioxidant	< 1
Colourant	< 1

There are two types of UV absorbers used in sunscreen formulations: physical absorbers and chemical absorbers. Physical absorbers block UV radiation from reaching the skin by absorbing, scattering or reflecting the incident light. Common examples of physical absorbers are titanium dioxide, iron oxide and zinc oxide. Chemical absorbers act by absorbing radiation and are excited to higher energy states. These high energy level species are unstable and hence deactivate to the ground state mostly by either radiative or nonradiative decay.

Some molecules deactivate to the triplet state, by intersystem crossing. From the triplet state they can deactivate to the ground state by radiative or nonradiative pathways. By absorbing and releasing energy, the absorbers prevent it from reaching the target site to cause damage. Besides decay some photochemical reactions such as scavenging of toxic oxidant species can take place **[113]**. Chemical absorbers fall into two categories depending on their absorptive properties: absorbers that absorb radiation in the UVA region (320 - 360 nm) and those that absorb in UVB region (290 - 320 nm). PABA absorbs in the region between 260 - 313 nm **[133]**, therefore it is a UVB absorber.

UV radiation has been implicated in the formation of free radicals and reactive oxygen species that damage the skin **[134, 135]**. Chemical absorbers and additives ameliorate the effect of UV in one of the following three ways **[136]**. Firstly, they can compete with the damaging agent (e.g. reactive species) for the target molecule. Therefore, agents like antioxidants compete with the reactive species for these target molecules. Secondly, by suppressing certain stages of the damage process but not offering complete protection, and lastly those that are able to repair the damage caused by UV **[136]**. They are able to carry out this function because of their absorptive properties and the photochemical reactions that ensue thereafter. They can quench excited triplets of other molecules **[85]**. Therefore, chemicals with properties that scavenge free radicals and reactive oxygen species can also provide photoprotection against cancer and photoaging **[137, 138]** and are worthy candidates for inclusion in sunscreen formulations. Such chemicals are generally referred to as antioxidants and are discussed in detail in the next section.

1.11 ANTIOXIDANTS IN SUNSCREEN FORMULATIONS

Antioxidant is a term often restricted to chain-breaking inhibitors. However, free radicals generated *in vivo* frequently damage proteins and DNA as well as lipids hence a broader definition has been introduced. "An antioxidant is any substance that when in low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of the substrate" **[139]**. In this context, a prooxidant is any substance that will hasten and enhance

oxidation of the substrate. These definitions cover almost everything found in living cells **[104, 140]**.

The scavenging of free radicals by antioxidants is by hydrogen atom donation **[134]**. However, if the ratio of the antioxidants to the substrate (such as the reactive oxygen species) is not optimal, the antioxidants generally show prooxidant effects at higher concentrations **[84]**. Reactive oxygen species (ROS), in particular, superoxide radical anion and singlet oxygen, have been implicated in UV radiation damage to skin. Quenching of singlet oxygen by antioxidants essentially follows the mechanism shown below **[134, 135]**:

$$LH + R^{\bullet} \longrightarrow L^{\bullet} + RH \qquad \text{chain initiation reaction}$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \qquad \text{chain propagation reaction}$$

$$LOO^{\bullet} + LH \longrightarrow L^{\bullet} + LOOH$$

$$LOO^{\bullet} + AH \longrightarrow A^{\bullet} + LOOH$$

$$L^{\bullet} + AH \longrightarrow A^{\bullet} + LH$$

$$LOO^{\bullet} + A^{\bullet} \longrightarrow LOOA$$

where $R^{\bullet}$ is a free radical produced by UV radiation and AH an antioxidant in the presence of a substrate (LH). LOOH is any organic molecule that has a hydrogen atom that could be abstracted.

The skin depends on antioxidants to reduce oxidative stress caused by ROS. In the process they are depleted and therefore need to be replenished both topically and physiologically **[5]**. Increased dietary intake of antioxidants has been shown to lessen UV-induced skin lesions **[141]**. When applied topically, free radical scavengers (antioxidants) in sunscreen formulations have been used to ameliorate the effect of oxidative stress **[142]** and are photoprotective **[137]**.

It has been reported that administration of antioxidants such as vitamin E and vitamin C can significantly reduce the incidence, and delay the progression, of various cancers such as skin cancer **[139]**. Antioxidants can also quench free radicals in the skin **[143]**. With this in mind, many antioxidants have been

included in sunscreen formulations with the aim of inhibiting UVB photocarcinogenesis **[144, 145]**.

1.11.1 VITAMIN E

Vitamin E is the generic term for tocopherol and tocotrienol homologues. Alpha-tocopherol, shown in Figure 1.14, is the most biologically active form of vitamin E **[86]**. It is the most abundant lipophilic antioxidant *in vivo* present in the skin **[146, 147]**. The concentration of vitamin E in dermal cells is 192.0 pmol mg^{-1} **[148]**. It has been implicated in a multitude of photoprotective roles in animal cells. *In vivo* it mainly functions as the major lipophilic antioxidant for biological systems by virtue of its ability to react with lipid peroxy radicals **[111, 149-151]**.

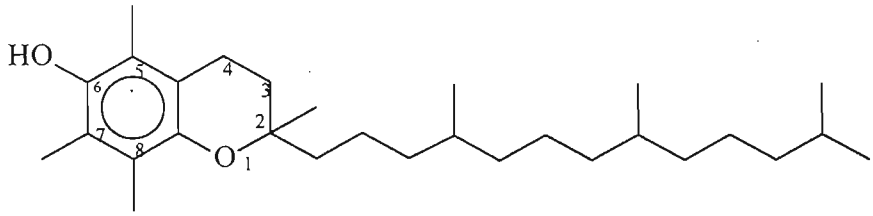

Figure 1.14: Structure of α-tocopherol, vitamin E.

Vitamin E has its UV absorption maximum at 290 nm for α-tocopherol and 284 nm for α-tocopherol acetate **[152]**. Although these values are on the edge of the UVB region, studies have shown that they can provide protection against UV-induced damage **[144]**. In fact tocopherols have received attention in sunscreen formulations as ingredients that prevent damage that is caused by over exposure to sunlight **[153]**. Since they absorb part of the UVB and are transparent to UVA, this implies that their absorption is only slightly responsible for their protective effect. Instead their ability to scavenge free radical and reactive oxygen species provides most of the protection **[153, 154]**. Another important factor that determines vitamin E's efficacy is its ability to penetrate the skin with ease **[154]**.

Studies have shown that topical application of vitamin E can inhibit the formation of cyclobutane photoproducts and it can especially lower thymine dimer yield in DNA in a UVB dose-dependant manner **[144]**. Vitamin E can also restore the level of thymidine in DNA in cells that have been exposed to UV radiation **[155]**. The mechanism by which vitamin E has an effect on thymine dimer formation is not clearly understood since the dimers result from direct absorption in the UVB region whereas vitamin E absorbs at the edge of this region **[86]**. Treatment by α-tocopherol can reduce the increase in erythema index (the amount of reddening of the skin) by 40-55% **[148]**. UVB sunscreen formulations that include 5% α-tocopherol in ethanol as an ingredient in the formulation are known to reduce tumour yields in skin **[156]**. Vitamin E also protects the skin from the harmful effects of UV-activated photosensitising dyes that damage skin tissues by generating $^1O_2$ upon UV irradiation **[148]**.

Finnen *et al.* **[157]** found that α-tocopherol and its acetate were effective in preventing erythema and edema that result from anthracine treatment in irradiated skin. Many studies indicate that derivatives of vitamin E are less effective in protection as compared with α-tocopherol **[154, 158]**. Nevertheless, vitamin E acetate is often used in sunscreens rather than vitamin E because it does not have the free phenol OH group and therefore oxidation does not affect it. It is supposed that hydrolysis of the ester takes place in the skin and leads to the photoprotection observed **[153]**. Alpha-tocopherol is relatively unstable in cosmetic formulations. Hence, to improve its stability, it is esterified and that is why vitamin E acetate is also used **[86]**.

1.11.2 VITAMIN C

Ascorbic acid (vitamin C) is the major hydrophilic antioxidant *in vivo* and acts as the first defense against free radicals in whole blood and plasma. It has been referred to as "an outstanding antioxidant in human plasma" **[159]**. In terms of concentration, it is the most predominant antioxidant in the skin being present at a concentration 200 times higher than that of vitamin E and 1000 times that

of ubiquinone, also an antioxidant **[86]**. The concentration of vitamin C in dermal cells is 283.1 pmol mg^{-1} **[146]**. Vitamin C levels in the skin are severely depleted after UV irradiation leaving the skin at risk for further damage by UV radiation **[141]**. Unlike other animals, human beings cannot synthesize vitamin C hence it has to be available in the diet or applied topically to maintain its level in the skin **[86, 104]**. Absorption of light by vitamin C is dependent on pH. Vitamin C has pK_1 and pK_2 as 4.17 and 11.57, respectively **[160]**. It has an intense absorption band with a maximum between 260 nm and 265 nm in neutral solution **[160, 161]**. The intensity of this band diminishes to half the original value in a few hours due to decomposition by oxidation that is not necessarily a result of irradiation. Nevertheless it is persistent at 245 nm when the pH is 3 or less **[160, 161]**.

Redox reactions are a major component of the reactivity of ascorbic acid. Ascorbate ion readily undergoes oxidation, forming an intermediate radical of low reactivity. This radical may account for many of ascorbate's antioxidant effects. A fairly reactive radical combines with ascorbate and a much less reactive radical is formed **[162]**. Standard reduction potential values indicate that ascorbate ion has relatively low reactivity when compared with other oxidizing radicals, i.e. it will tend to quench more reactive oxygen species **[163]**. Ascorbic acid scavenges aqueous radicals more efficiently than it does lipophilic radicals **[134]**. It is thus a powerful reducing agent in aqueous media but the reducing properties are less evident in non-aqueous media. Figure 1.15 shows ascorbic acid and the intermediates formed during oxidation.

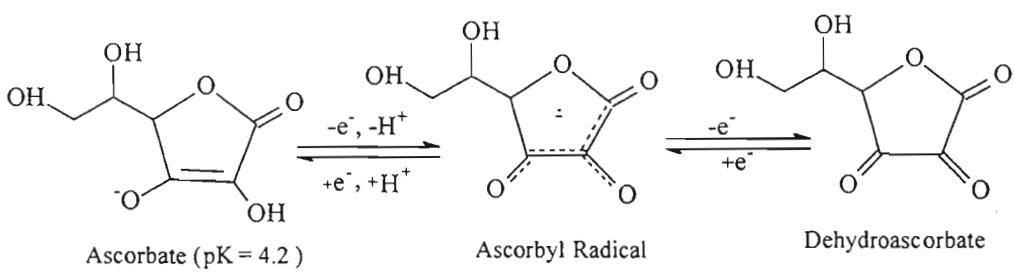

Figure 1.15: Oxidation products of ascorbic acid **[164]**.

Reactive oxygen species, like singlet oxygen partly account for the damage caused by UV radiation on the skin **[141]**. Studies have shown that ascorbic acid can neutralise singlet oxygen **[86, 165 - 167]**. Topical application of vitamin C has been shown to provide protection of the skin from UVB damage as measured from erythema **[141]**. This photoprotective property of vitamin C qualifies it as an active sunscreen ingredient in sunscreen formulations **[144, 145]**. To achieve delivery to the skin, the pH of a formulation that contains ascorbic acid must be kept below 3.5 in order to ensure protonation of the ascorbic acid, which is important for its antioxidant activity **[86]**.

1.11.3 COMBINED PRESENCE OF VITAMINS E AND C IN SUSNCREEN FORMULATIONS

As described earlier, vitamins E and C have individually been used as ingredients in sunscreen formulations. Both vitamins are depleted on interaction with UVB radiation **[144, 145]**. The regeneration of vitamin E by ascorbate has been assumed to be responsible for the synergistic protective effect of vitamins E and C **[168]**. Krol *et al.* **[144]** have suggested that the photoprotective properties of vitamins E and C are due to both their antioxidant and UV-absorptive properties. These factors have promoted the development of sunscreens that contain both vitamins C and E. Goodall and Hoffer **[169]** have recommended that vitamin C and vitamin E be used as protection against the damages of excessive ultraviolet radiation exposure. They suggest a daily dose of 3 grams or more of vitamin C and 800 IU of vitamin E. Combined topical application of 15% ascorbic acid and 1% vitamin E has been shown to provide 4-fold protection against UV-induced erythema and thymine dimer formation **[86]**. Placzek *et al.* **[170]** showed that a combined oral intake of vitamin E and vitamin C significantly reduced thymine dimer formation as a result of UVB exposure. Although, in these studies, the vitamins were not applied topically, they could have a bearing on sunscreen formulations that incorporate them. Bissett *et al.* **[171]** have shown that topically applied vitamin E and vitamin C is effective in moderating low level, chronic UVB but not UVA damage to mouse skin. Depending on the ratio of the two vitamins there can be depletion of vitamin E by vitamin C, or vice versa **[104, 172]**. Studies have indicated that

although vitamin C is needed more than vitamin E for photoprotection against UVB radiation, the latter is consumed more than the former which is indicative of the fact that when together, vitamin E provides the larger share of the protection **[173, 174]**. Although ascorbic acid and α-tocopherol are individually prone to oxidation in solution, combing the two helps stabilize the formulation **[86]**. They are both effective at very low concentrations after topical application in preventing UV-induced immunosupression **[175]**.

1.12 VITAMIN E AND SINGLET OXYGEN

Certain chromophores in biological systems like the skin can photosensitise the formation of singlet oxygen on irradiation with UV radiation **[176]**. Vitamin E is known to react with singlet oxygen that has been produced by a photosensitiser, by physical quenching and chemical reaction **[177]**. Therefore, vitamin E can protect the skin form this harmful effect of UV radiation **[148]**. There are four isomers of tocopherols designated as α, β, γ and δ. Quenching of singlet oxygen by α-tocopherol is the most effective when compared with other tocopherols **[111, 93]**. Therefore, this discussion will center on the chemistry of α-tocopherol. Henceforth, when we refer to vitamin E, we will be implying α-tocopherol or its acetate and vice versa. α-Tocopherol can also quench the reactive intermediates that would normally bind to and damage epidermal DNA **[178]**.

Stevens *et al.* **[179]** have proposed a mechanism by which α-tocopherol quenches singlet oxygen in the presence of a self-photosensitiser, P. The scheme represents both physical and chemical quenching. α-Tocopherol can quench the triplet sensitiser, which is the precursor of singlet oxygen $^1O_2$ and thymine dimer formation. The relevant steps in the quenching mechanism are summarised below:

$$P + h\nu \longrightarrow {}^1P^* \qquad 1$$

$${}^1P^* + {}^3O_2 \longrightarrow {}^3P^* + {}^3O_2 \qquad 2$$

$${}^3P^* + {}^3O_2 \longrightarrow P + {}^1O_2^* \qquad 3$$

$${}^3P^* + E \longrightarrow P + E \qquad 4$$

$$P + {}^1O_2^* \longrightarrow PO_2 \qquad 5$$

$$E + {}^1O_2^* \longrightarrow \text{quenching} \qquad 6$$

$${}^1O_2^* \longrightarrow {}^3O_2 + h\nu \qquad 7$$

where E stands for vitamin E and PO_2 is as described in Section 1.8. Step 6, representing the quenching of singlet oxygen, is essentially 99% a physical process **[179]**.

$$E + {}^1O_2 \longrightarrow {}^3E + {}^3O_2 \qquad 8$$

Since the triplet energy for α-tocopherol is not expected to lie below the energy of singlet oxygen at 95.707 kJ mol^{-1}, energy transfer as shown above in step 8 is very unlikely to take place. The change in spin angular momentum requirement is that either a charge transfer or a biradical intermediate is required **[179]**. In fact, a charge-transfer mechanism involving an intermediate has been proposed in the quenching process **[111, 180]**. Fahrenholtz *et al.* **[149]** have proposed a similar mechanism. Chemical reaction involves photooxidation with the main products being α-tocopherol quinone and α-tocopherol quinone epoxide **[111, 181]**. These photoproducts are derived from endoperoxide that results from 1,4-cycloaddition of the aromatic ring **[151]**.

The quenching and antioxidant properties of vitamin E result from the chemistry of the two aromatic rings shown in Figure 1.14 **[111]**. Esterification of the hydroxyl functional group at position 6 in the chromane ring removes the quenching ability of tocopherols indicating that a free hydroxyl is necessary for quenching of singlet oxygen **[111]**. Figure 1.16 shows α-tocopherol esterified with an acetate to give α-tocopherol acetate.

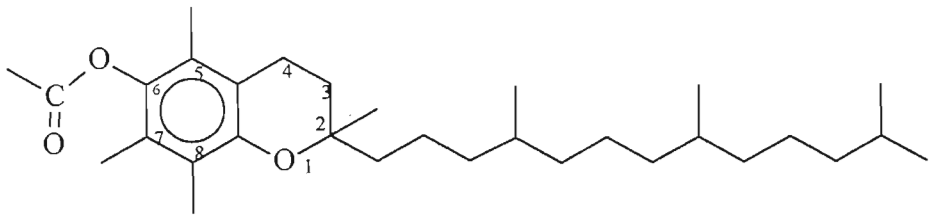

Figure 1.16: Structure of α-tocopherol acetate.

Trolox, a water-soluble analogue of vitamin E that does not contain the phytyl chain, shows the same quenching ability as α-tocopherol suggesting that the chain does not contribute to the quenching of singlet oxygen nor does it have any antioxidant properties **[111]**. The position of the methyl group on the chromane ring of vitamin E affects the type of quenching. Methylation of position 5 in the chromane ring of vitamin E enhances physical quenching whereas methylation on position 7 enhances chemical reactivity **[111]**. This is based on the fact that β-tocopherol with two methyl groups at position 5 and 8 has the same physical quenching ability as α-tocopherol, which has methyl groups at positions 5, 7 and 8. β-tocopherol shows no chemical reactivity at all whereas both types of quenching are observed in α-tocopherol **[111]**. The rate constants for physical quenching and chemical reaction for α-tochopherol with singlet oxygen are 2.80×10^8 M^{-1} s^{-1} and 3.6×10^6 M^{-1} s^{-1} respectively in a 1:1 mixture of deuterated water and ethanol **[111]** and 6.2×10^8 M^{-1} s^{-1} and 4.6×10^7 M^{-1} s^{-1} respectively in methanol with a total rate constant equal to 6.7×10^8 $M^{-1}s^{-1}$, making it an effective scavenger for singlet oxygen **[150]**. From these rate constants and the many references on quenching of singlet oxygen by α-tocopherol, it is evident that physical quenching represents a bigger percentage of the quenching process **[111, 149-151]**. One molecule of α-tocopherol has been shown to be able to deactivate 120 molecules of singlet oxygen by

physical quenching for every molecule of singlet oxygen that is deactivated by reaction **[149]**. Figure 1.17 is a schematic diagram showing deactivation of singlet oxygen by α-tocopherol. The extent of physical quenching or chemical reaction is dependent on the nature of the solvent. For example, the polarity of the solvent plays an important role **[111, 180, 181]**. The activity of tocopherol is also concentration dependant and sometimes temperature dependent **[182]**. During irradiation α-tocopherol is thought to lose some of its singlet oxygen quenching ability by chemically reacting with singlet oxygen **[181]**.

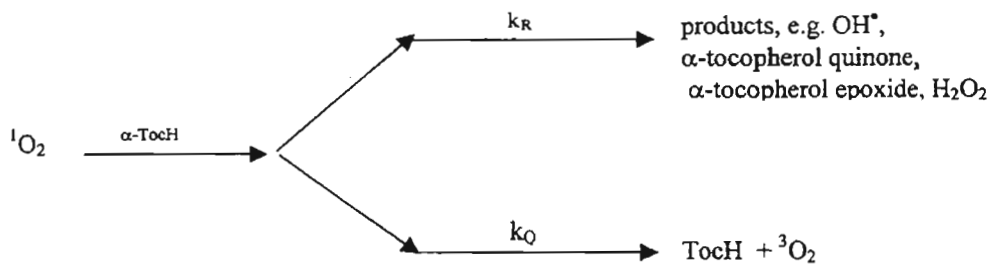

Figure 1.17: Quenching of singlet oxygen by α-tocopherol **[181]**.

From the preceding paragraphs, it is evident that vitamin E has a positive contribution in the fight against the ailments caused by UV radiation. Nevertheless, it has been suggested that in skin, vitamin E can act as an endogenous photosensitiser on absorption of UV radiation and thus is converted to its radical form which is able to deplete other antioxidants or attack lipid molecules to give the tocopheryl radical. This is the prooxidant part of vitamin E **[183, 184]**.

The regeneration of vitamin E from the radical is the one responsible for the depletion of other antioxidants. Therefore, it appears that there is a conflict in the functions of α-tocopherol. However, the beneficial antioxidant and absorptive properties appear to outweigh the harmful effects **[183]**. The photoprotective effect of vitamin E is not entirely attributable to itself alone but to its involvement with other antioxidants like vitamin C **[148]**.

1.13 VITAMIN C AND SINGLET OXYGEN

Vitamin C can scavenge singlet oxygen formed by a photosensitiser, P **[185]**. Nevertheless, a photosensitiser can also exert some stress on the antioxidant activity of vitamin C and hence lower its effective ability to quench singlet oxygen **[177]**. A triplet photosensitiser formed by absorption of light can react with ascorbate ion to give the ascorbyl radical thus depleting ascorbic acid, which provides the ascorbate ion necessary to quench singlet oxygen. Singlet oxygen can react with the ascorbate ion as shown in 1.10 **[177]**.

$$^3P + AsH^- + {}^3O_2 \longrightarrow As^{-\cdot} + P + O_2^- + H^+ \qquad 1.9$$

$$AsH^- + {}^1O_2 \longrightarrow \text{hydroperoxy dehydroascorbic acid} \qquad 1.10$$

where AsH^- represents the monovalent ascorbate ion and $As^{-\cdot}$ represents the ascorbyl radical.

Davies *et al.* **[166]** have shown that irradiation of a mixture of a photosensitiser like methylene blue and vitamin C results in the uptake of molecular oxygen and that the absorbance of the photosensitiser remains constant. The constant absorbance value for the photosensitiser suggests that it is not consumed in a chemical reaction but instead is involved in a quenching process. They proposed and confirmed two mechanisms that occur at the same time and are consistent with photosensitiser regeneration and the consumption of singlet oxygen by vitamin C. The first one involves energy exchange between the species taking part and the second one involves a radical mechanism **[166]**.

Mechanism 1

$$P + h\nu \xrightarrow{I} {}^1P \quad (1)$$

$${}^1P \xrightarrow{k_{isc}} {}^3P \quad (2)$$

$${}^3P \xrightarrow{k_o} P \quad (3)$$

$${}^3P + {}^3O_2 \xrightarrow{k_1} P + {}^1O_2 \quad (4)$$

$${}^1O_2 + AsH_2 \xrightarrow{k_2} AsH_2O_2 \quad (5)$$

$${}^1O_2 \longrightarrow {}^3O_2 \quad (6)$$

Mechanism 2

$${}^3P + AsH_2 \xrightarrow{k_4} AsH^{\bullet} + PH^{\bullet} \quad (7)$$

$$PH^{\bullet} + {}^3O_2 \xrightarrow{k_5} P + HO_2^{\bullet} \quad (8)$$

$$AsH^{\bullet} + {}^3O_2 \xrightarrow{k_6} As + HO_2^{\bullet} \quad (9)$$

$$2HO_2^{\bullet} \xrightarrow{k_7} H_2O_2 + {}^3O_2 \quad (10)$$

where AsH_2, AsH_2O_2, $AsH^{\bullet}$, $PH^{\bullet}$ and $HO_2^{\bullet}$ are the divalent ascorbic acid, hydroperoxy dehydroascorbic acid, ascorbate radical, a reduced radical of a photosensitiser and hydroperoxyl radical respectively.

The same group of researchers found that the rate of oxygen uptake, and hence singlet oxygen formation, increases with increase in irradiation time. When using riboflavin as a sensitiser, Kim *et al.* **[186]** showed that the triplet photosensitiser could react directly with ascorbate ion according to the following reaction:

$${}^3P^{*} + AsH^{-} + {}^3O_2 \longrightarrow As^{\bullet -} + P + O_2^{\bullet -} + H^{+}$$

which is essentially the sum of reactions 7 and 8 as proposed by Davies *et al.* **[166]**. There are many contradicting literature values on the rate constant for quenching of singlet oxygen by vitamin C in biological systems **[94]**. The rate constant for quenching of singlet oxygen by vitamin C in aqueous solution lies between 2.5×10^6 and 1.6×10^8 M^{-1} s^{-1} clearly indicating that the rate constant depends on the experimental conditions **[167]**. For example, the value for the

rate constant for quenching of singlet oxygen by ascorbate has been quoted as 1 x 10^7 M^{-1} s^{-1} **[187]**. This value includes both deactivation by chemical reaction and physical quenching. Kuznestova *et al.* **[165]** found a value of 6.8 x 10^8 M^{-1} s^{-1} for the rate constant with chemical reaction being 20 times lower than physical quenching. They also found that oxidation of ascorbic acid by singlet oxygen in the presence of a photosensitiser is pH-dependent. Protonation of vitamin C depends on pH and hence its rate constant for singlet oxygen quenching will also be pH-dependent **[167]**. The quenching of singlet oxygen in solution is essentially due to reaction with ascorbate radical, which is a better reductant than ascorbic acid **[167]**.

Although vitamin C can quench singlet oxygen, Linetsky *et al.* **[145]** found that irradiation of ascorbic acid-glycated proteins yielded singlet oxygen as the major product. This implies that there is a possibility that despite the fact that ascorbic acid quenches singlet oxygen, it can also form it. While using N,N-dimethyl-4-nitrosoaniline (RNO) to monitor singlet oxygen formation, they found that its formation increased linearly with increase in irradiation time **[145]**. Trommer *et al.* **[188]** reported that vitamin C is a prooxidant in a concentration-dependent manner and that its degradation was detected after the prooxidation.

There have been conflicting reports on the interaction of vitamin C and ROS. Both antioxidant and prooxidant properties have been reported **[159, 188]**. Recently, vitamin C's genotoxic effect has been suggested based on its ability to produce secondary products that can damage DNA **[189]**. Although it is a valuable free radical quencher, it may itself be susceptible to UV-mediated destruction **[141]** or even cause damage to DNA **[190]**. It can, *in vitro,* exert prooxidant properties. For example in the presence of copper ion and hydrogen peroxide, it causes severe oxidative damage to the bases of DNA **[164]**.

1.14 COMBINED ANTIOXIDANT AND PROOXIDANT ACTIVITIES OF VITAMINS E AND C

As discussed earlier, both vitamins C and E exhibit antioxidant activity individually. This property is a positive aspect of the two vitamins. Nevertheless, some findings have revealed that both vitamins C and E could be prooxidants either individually or together **[86]**. In general, antioxidants show prooxidant properties at high concentrations **[84]**. Antagonism refers to a situation where two antioxidants boost one another's prooxidant activity, i.e. combined prooxidant activity **[134]**. Synergism refers to the situation when two antioxidants boost one another's activity **[134]**. A number of factors such as concentration, interaction between the vitamins, their combined induction periods (lag phases) and temperature, determine whether antioxidant properties or prooxidant properties will be exhibited **[134, 182]**. The following paragraphs discuss the individual prooxidant properties of vitamin C and E besides the synergism and antagonism that arise due to their combination.

Controversies regarding the interaction of vitamin C and reactive oxygen species as antioxidant or prooxidant have been reported **[86]**. Vitamin C exhibits prooxidant activity as it autoxidises to dehydroascorbic acid (see Equation 1.10) **[191]**. Hu *et al.* **[192]** showed that ascorbate ion enhances DNA damage in a concentration-dependent manner and Jenkins *et al.* **[190]** showed that vitamin C causes DNA damage at very high concentration. A conflicting finding indicates that vitamin C acts as an antioxidant in high concentration and as a prooxidant in low concentration **[193]**.

Vitamin E can also show prooxidant properties. There have been suggestions that vitamin E in the skin could act as a photosensitiser on absorption of UV radiation while at the same time being converted to its radical which exhibits prooxidant activity by depleting other antioxidants **[183]**. Studies have shown that it can lose its antioxidant activity at high concentration and become a prooxidant **[194]**.

It has been shown quantitatively by Sharma *et al.* **[195]** that there is interaction between the two vitamins on exposure to UV radiation. Both vitamin C and E behave differently when they are alone and when they are combined. They can lose their antioxidant ability, become prooxidants, or show synergism or antagonism. The ratio of vitamin E to vitamin C concentration has been shown to determine which properties prevail **[164]**. The tendency for vitamin C to be a prooxidant in the presence of vitamin E increases with increases in interaction between the two and if a photosensitiser is also present **[177]**. For example, in the presence of vitamin E, the rate of ascorbate oxidation is enhanced by riboflavin as a photosensitiser **[177]**.

Slater *et al.* **[196]** showed that synergism exists between vitamin E and vitamin C. They found that addition of vitamin C reduces the α-tocopheryl radical back to α-tocopherol, i.e. there is an increase in the amount of α-tocopherol. Packer *et al.* **[197]** measured the rate constant for this reaction and found it to be 1.55 x 10^{-6} M^{-1} s^{-1}. This has been verified by electron spin resonance (ESR) studies **[198]**. Regeneration of vitamin E by vitamin C is shown in Figure 1.18. It has also been shown that addition of vitamin C to a system containing vitamin E depletes the latter and that vitamin C can also be depleted on addition of vitamin E **[177, 196]**.

The synergism is due to the regeneration of α-tocopherol by reduction of its tocopheryl radical by the ascorbate ion, AsH^-. α-Tocopherol traps peroxy radicals or any free radical, to give the α-tocopheryl radical. It is then reduced by vitamin C, the ascorbate ion, to give α-tocopherol while the ascorbate is ultimately oxidized to semidehydroascorbate radical. Two molecules of this radical can combine and be reduced as follows **[199]**:

$$ROO^{\bullet} + \alpha-TOH \xrightarrow{K_R} ROOH + \alpha-TO^{\bullet} \quad 1$$

$$\alpha-TO^{\bullet} + AsH^- \longrightarrow \alpha-TOH + A^{-\bullet} \quad 2$$

$$As^{-\bullet} + As^{-\bullet} \xrightarrow{H^+} As + AsH^- \quad 3$$

where ROOH, ROO$^{\bullet}$, α-TOH, α-TO$^{\bullet}$, AsH^{-} and $As^{-\bullet}$ represent an organic peroxide, organic peroxyl radical, α-tocopherol, tocopheroxyl radical, monovalent ascorbate ion and ascorbate free radical, respectively.

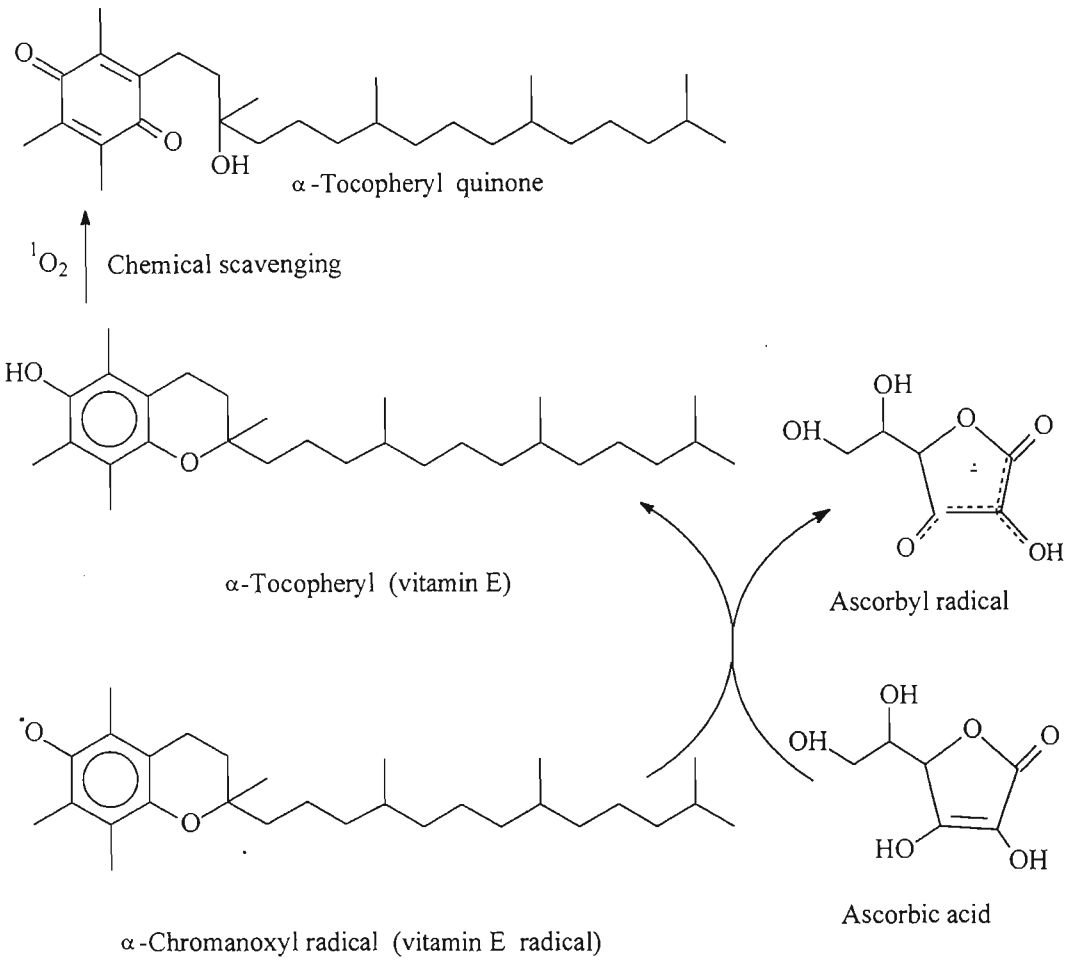

Figure 1.18: Chemical scavenging of singlet oxygen by α-tocopherol and its regeneration from α-tocopherol radical by ascorbic acid **[148]**.

There is a lot of evidence, which indicates that there is a synergistic antioxidant interaction between vitamin E and vitamin C in a wide variety of *in vitro* models **[134]**. Some *in vitro* studies have suggested that vitamin E may be recycled by interaction with ascorbate **[168]**. In the presence of vitamin E and even copper ions, vitamin C delays the oxidation of a substrate by recycling α-tocopherol. Once the oxidation of the substrate is underway and presumably all

the α-tocopherol has been oxidized, vitamin C can accelerate the oxidation. This implies that the ratio of α-tocopherol to vitamin C will determine which of the two properties of vitamin C, antioxidant (hence synergism) or prooxidant (hence antagonism) will prevail. In most cases the former prevails over the latter **[164]**. Synergism between the two vitamins was shown to be at its best at a concentration of 5 mM of each **[200]**. Besides concentration, for two antioxidants to act synergistically, their induction period should be longer than the sum of the induction periods of the individual antioxidants **[134]**. The combination of the two antioxidants is effective especially in low oxygen concentration **[169]**.

1.15 OBJECTIVES OF THIS RESEARCH WORK

As mentioned earlier, when PABA is applied in combination with vitamin C, it can reduce the number of sunburn cells, i.e. reddening of the skin **[138]**. Sunburn is the inflammation and blistering of the skin as a result of overexposure to sunlight. The minor beneficial properties of PABA as an active sunscreen ingredient have been surpassed by its harmful effects. Its ability to penetrate human cells and photosensitise dimer formation is well established **[66]**. PABA-photosensitised formation of singlet oxygen has also been shown to occur **[120]**. These two factors are very important in carcinogenesis, especially in skin cancer, and aging. PABA has its highest triplet quantum yield at pH 3 **[65]**. Triplet PABA is an important factor in the dimerisation process **[65]**. The photoproducts of the irradiation of PABA together with thymine and the formation of singlet oxygen depend on the concentrations of the photosensitiser and the reactants and also on the photon flux. The two antioxidants, vitamin E and vitamin C are also included in sunscreen formulations. They have been shown to scavenge singlet oxygen and also inhibit formation of pyrimidine photoproducts. Their activities are also concentration dependent. Vitamin C is most stable in aqueous solution at pH 3. This is the pH where it is fully protonated. Because of the properties of vitamin C and PABA in aqueous solution, it is therefore necessary to maintain the solution to be irradiated at pH 3 so that only one of the two forms of PABA predominates and that the properties of vitamin C do not change. Irradiation of

the reagents should be at wavelengths greater than 300 nm so as to ensure that the dimerisation is via the photosensitised route. Another reason is that wavelengths shorter than 300 nm do not reach the earth's surface from the sun. α-Tocopherol is more effective in terms of its vitamin E activity than its acetate. Nevertheless, the acetate was the reagent of choice for our investigation because it is known to hydrolyse in the skin and give similar results as the free tocopherol and has the advantage of being soluble in water at 25 °C. Thymine, a base in DNA has also been shown to interact with singlet oxygen. We therefore postulate that the scavenging and absorptive properties of the antioxidants could counter the deleterious effects of PABA.

The objective of this research work was to investigate our postulate, i.e. to determine the effect of the antioxidants vitamins E and C on the PABA-photodimerisation of thymine and on the formation of singlet oxygen in aqueous solution at pH 3.0 To achieve this objective, concentrations of the photosensitiser, PABA, and the substrates and photon flux had to be varied and the reaction medium maintained at pH 3.0. Sunscreen formulations typically have a pH between 5 and 8 but the chemistry of vitamin C and that of PABA required the pH be at 3.0. Irradiation of solutions was carried out at wavelengths greater than 300 nm that simulated solar terrestrial radiation so that the results could be more relevant to sunscreen photochemistry.

The equipment and experimental techniques used to acquire the data are described in Chapter 2. The results obtained are presented and discussed in Chapter 3. Chapter 4 draws conclusions from the results obtained and gives suggestions for further research work.

CHAPTER 2

EXPERIMENTAL

This chapter, describing the experimental work performed, falls into two main parts. The first part describes the analysis techniques used to obtain data for thymine dimer yields in irradiated solutions containing the photosensitiser, PABA, in the presence or absence of antioxidant(s). This is covered in Sections 2.3 to 2.8. The second part details the investigation of the PABA-photosensitised formation of singlet oxygen in the presence of thymine and the antioxidants. This is covered in Section 2.9.

2.1 MATERIALS

The chemicals and materials used in this work, plus the grades of the reagents and manufacturer's information, are listed in Appendix A.

2.2 EQUIPMENT

The equipment used for spectroscopic, photoproduct and singlet oxygen analyses is listed in Appendix B.

2.3 LIGHT SOURCE AND IRRADIATION TECHNIQUES

This section deals with the light source and techniques used for the irradiation of various samples investigated in this work.

Investigation into the PABA-photosensitised formation of thymine dimer and singlet oxygen and the effect of antioxidants thereon required the use of a light source of high intensity. For this purpose, an Osram HBO 500 W high pressure mercury lamp (Figure 2.1), powered by a Schrieber DC supply, was suitable. The lamp produces UV radiation of high intensity with extensive pressure broadened mercury emission lines superimposed on a continuum. The relative

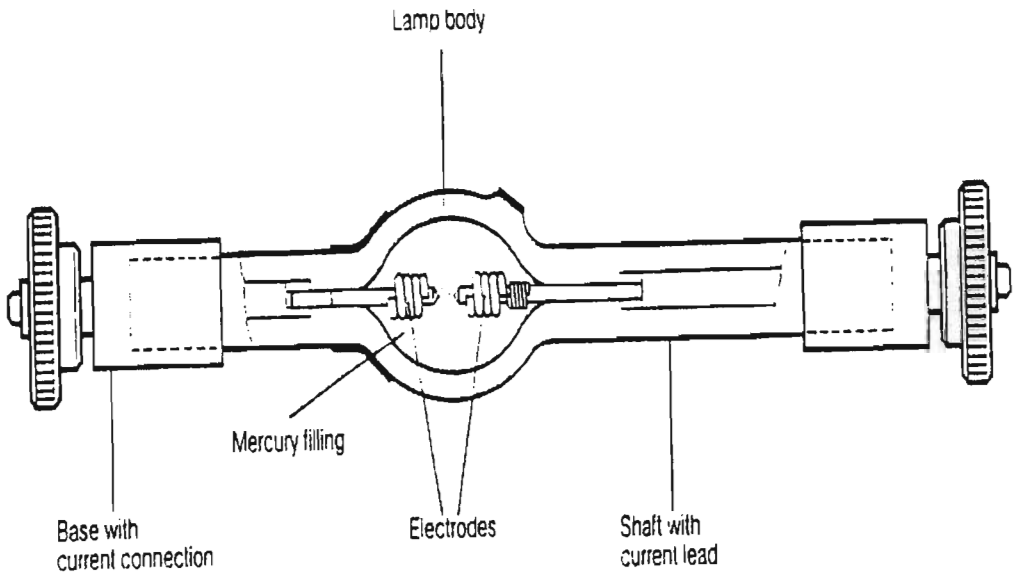

Figure 2.1: Osram HBO 500 W high pressure mercury lamp **[74]**.

spectral distribution of intensities, as shown in Figure 2.2, illustrates that together with appropriate filters, it is suitable for irradiation in the UVB (280-315 nm) and UVA (315-400 nm) wavelength ranges. Previous members of this research group have provided descriptions of the lamp housing extensively but a brief description is given here **[56, 77]**. It consists of an insulated steel box in which the lamp is fixed and connected to the power supply. The box has a cooling fan to prevent damage of the filter due to the heat produced by the lamp. Any ozone produced by UV radiation is removed by use of an extractor fan fitted above the lamp housing. To allow radiation to the photolysis cell, there is a circular aperture in front of the lamp. An external cradle consisting of a shutter gate, a filter holder and a cell holder is fixed to the aperture as shown in Figure 2.3. The manufacturer recommends that, for optimum results from the lamp, it should not be used when it has exceeded a lifetime of 400 hours. For any quantitative work, the intensity of the lamp needs to settle. At least a 5-hour interval between when it was last switched off and when switching on was thus necessary. A warm-up period of 15 minutes was allowed before use to allow the lamp to attain a maximum and stable intensity. A Blak-Ray J-221 long wave photovoltaic UV intensity meter was used to monitor the lamp intensity. A logbook was maintained for recording the lamp intensity and the

number of hours the lamp had been operated. This gave an up-to-date record of the remaining lifetime as shown in Figure 2.4.

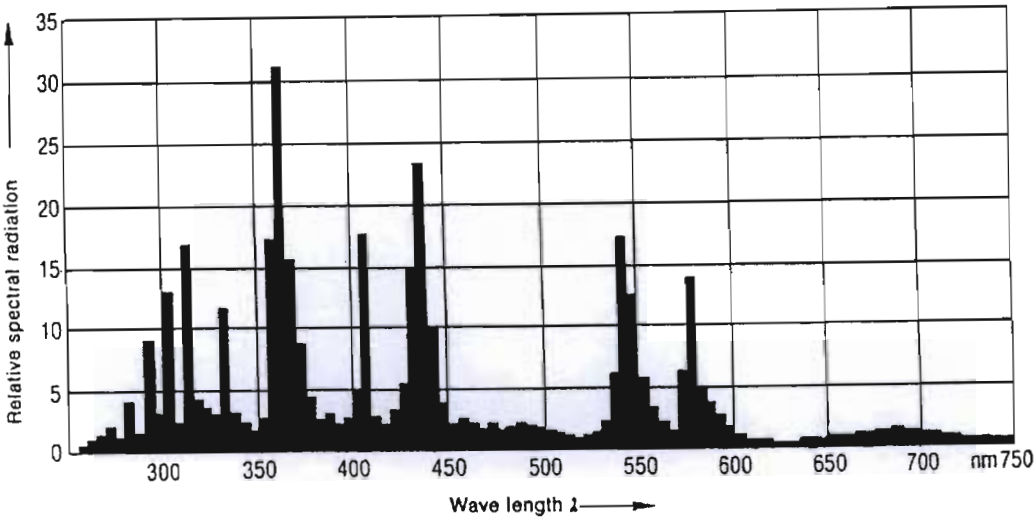

Figure 2.2: The relative spectral distribution of the Osram HBO 500 W high pressure mercury lamp **[74]**.

From Figure 2.2, it can be seen that the Osram HBO 500W lamp emits radiation in the wavelength range of about 250 nm to 750 nm. The investigation in this work required simulating solar radiation incident on the earth's surface. For this reason a suitable filter had to be used to cut off the light energy. A 10 mm thick Pyrex filter was used for this purpose. It allowed only wavelengths greater than 300 nm to pass through. The transmission characteristics of this filter are shown in Figure 2.5. This filter was used for all the irradiations performed in this work.

For thymine dimerisation experiments, irradiations were carried out in a 1-mm pathlength quartz cuvette. The cuvette was covered with either a Teflon stopper or with the stopper and parafilm in order to prevent evaporation of the contents. The cell is shown in Figure 2.6. The photolysis cell could hold only 480 μL of thymine solution. The nitrogen-purged solution was transferred to the cell using a micropipette. It was then stoppered and sealed with parafilm to prevent evaporation. The cell was thereafter positioned in the optical train. For short irradiation times the cell was not covered with parafilm because there was no danger of evaporation and loss of sample. Irradiations of solutions for singlet

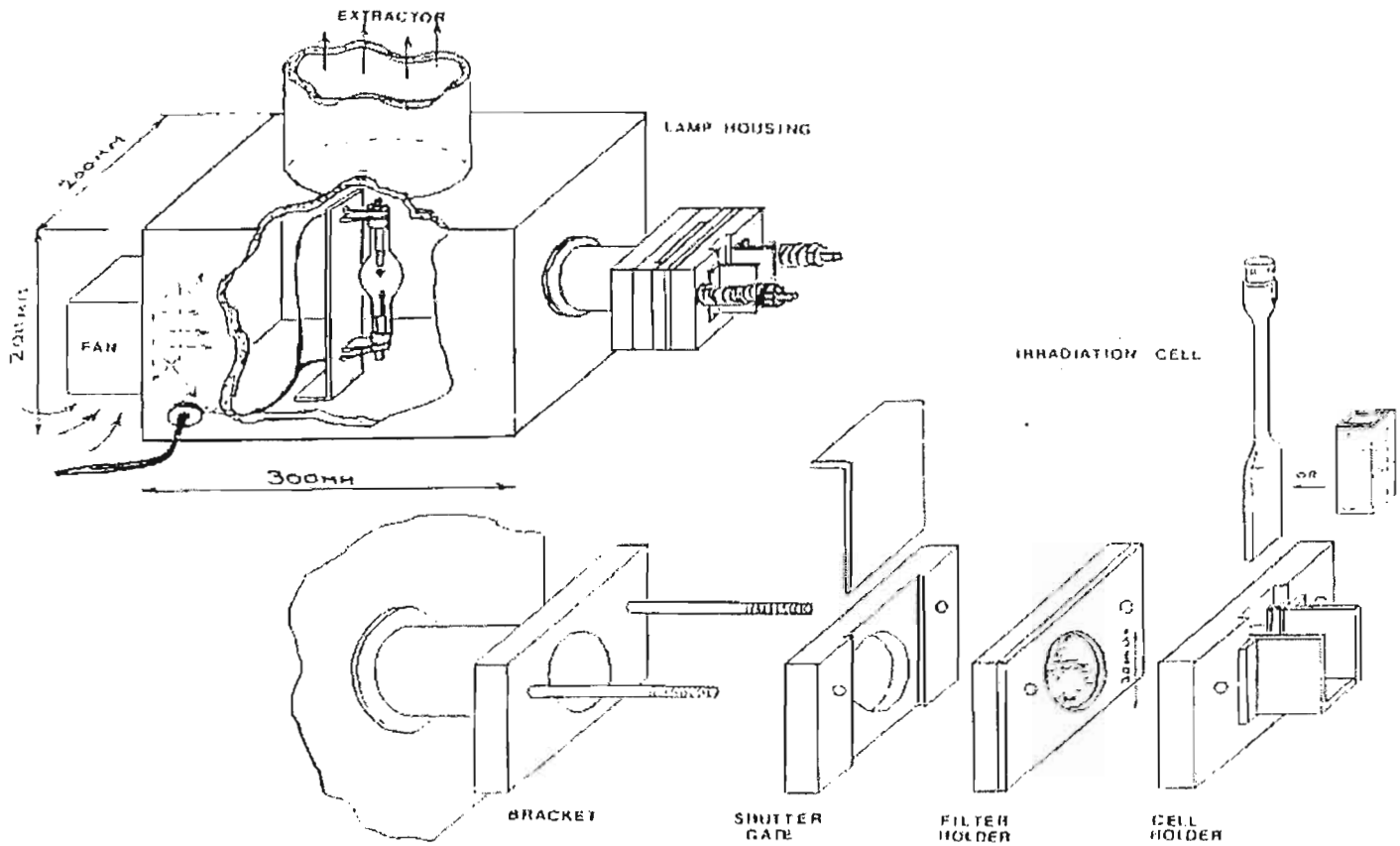

Figure 2.3: The Osram HBO 500 W high pressure mercury lamp housing **[74]**.

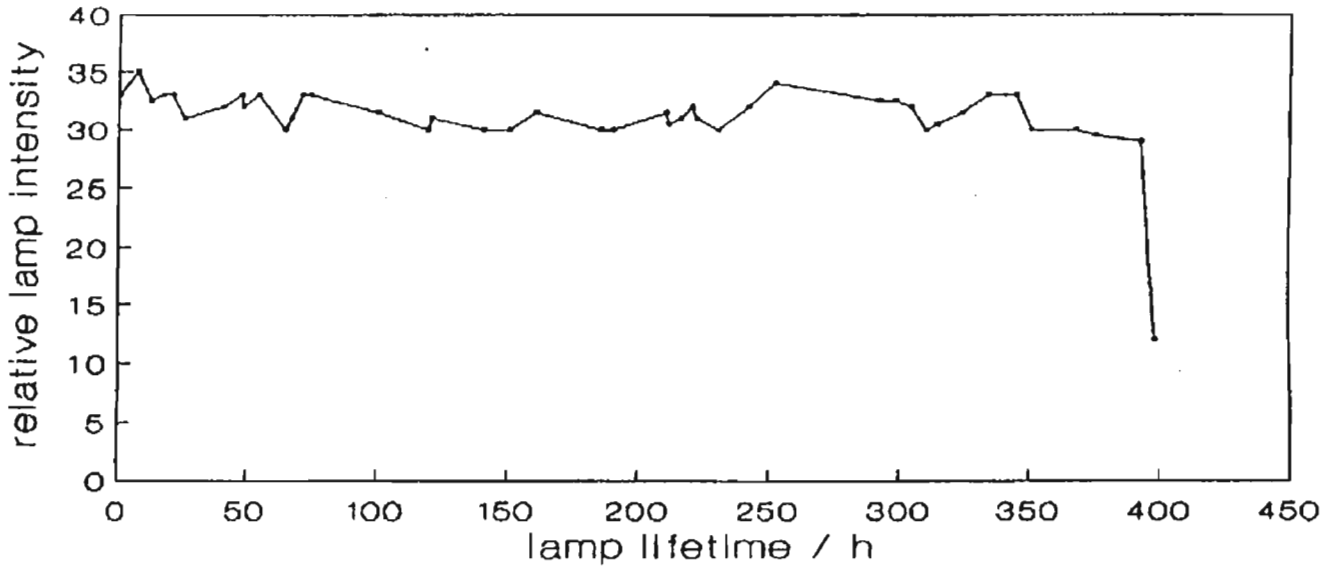

Figure 2.4: Variation of the relative intensity of the Osram HBO 500 W high pressure mercury lamp with time **[74]**.

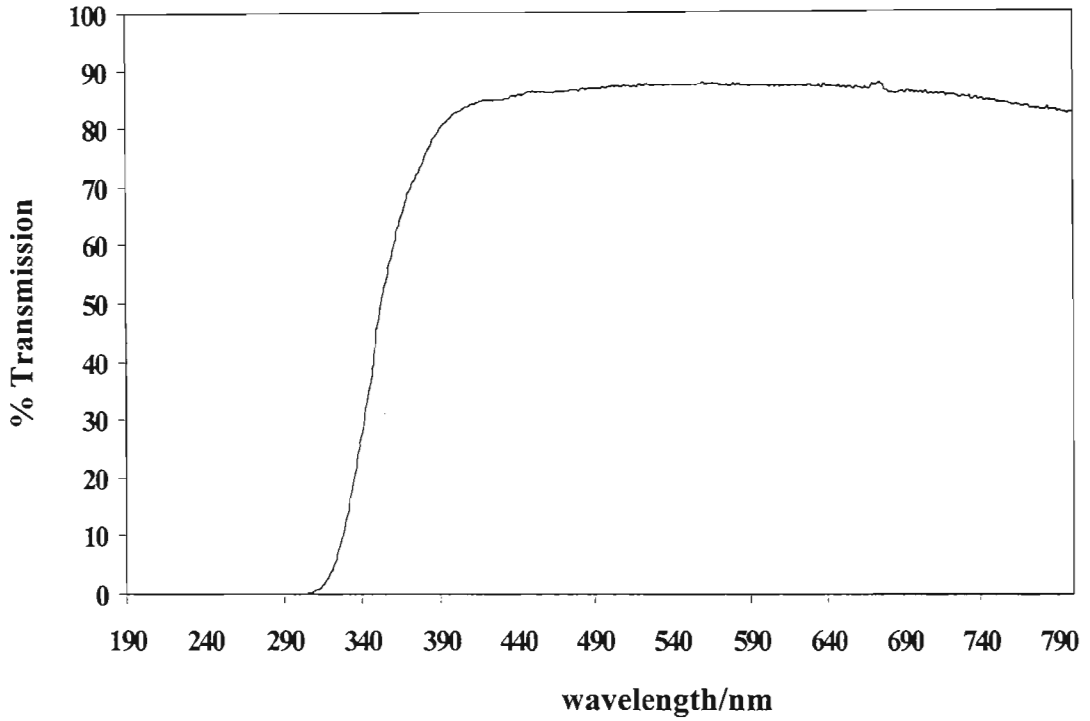

Figure 2.5: Transmission characteristics of the 10-mm thick Pyrex filter.

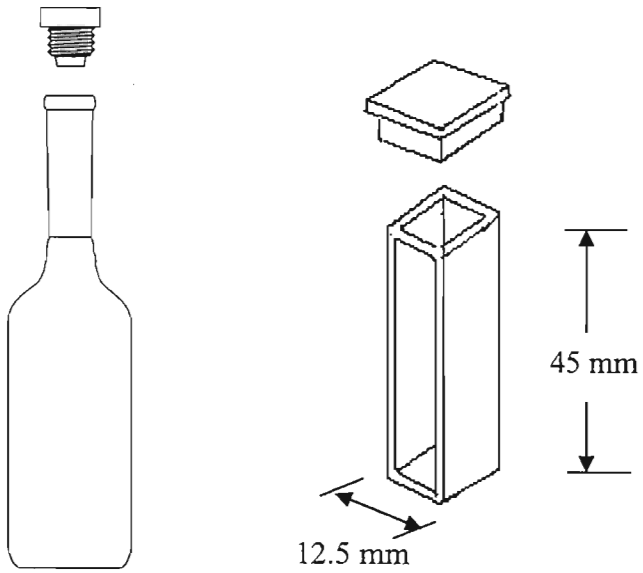

Figure 2.6: Quartz cuvettes of 1 mm and 10 mm pathlength for irradiation and absorbance measurements.

oxygen studies were done with a 10 mm pathlength quartz cuvette. After filling the cuvette with the solution it was then positioned in the optical train and covered with the lid. No parafilm was used since the irradiation times were short.

Other than solutions for singlet oxygen studies, samples were purged with nitrogen gas for fifteen minutes before irradiation to remove any dissolved oxygen that could quench the triplet excited state of thymine since quenching by oxygen reduces thymine dimer yield **[34, 41, 43]**. There is evidence that oxygen molecules can be strongly absorbed on the surface of the cell **[201]**. The purging saturates the solution with nitrogen thus reducing dissolved oxygen and ensuring uniform mixing of thymine and other substrates. The apparatus used for purging the solution with nitrogen is shown in Figure 2.7. Nitrogen was first saturated with some of the solution prepared then finally passed into the solution to be irradiated. The investigation of singlet oxygen formation required an environment saturated in oxygen.

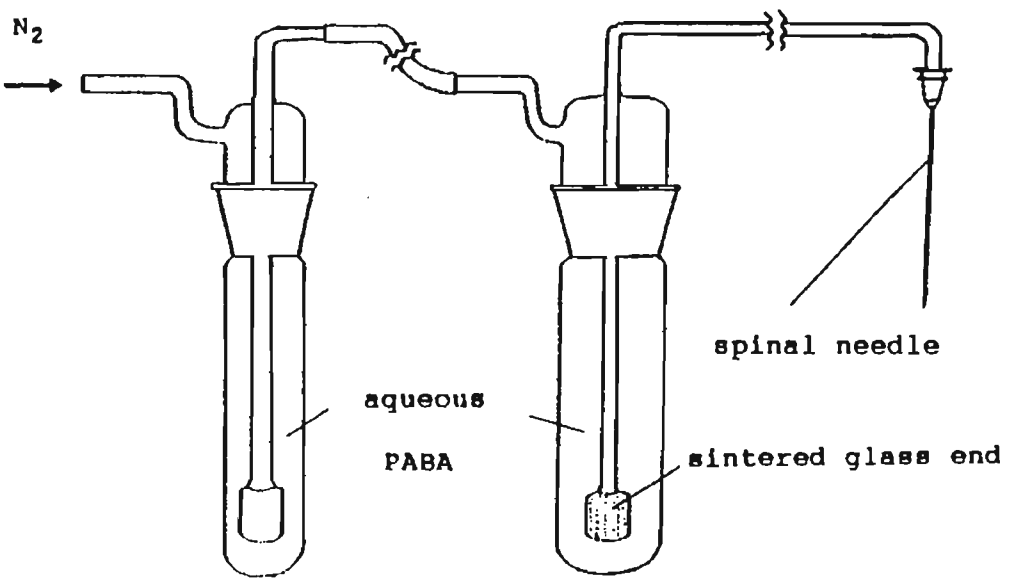

Figure 2.7: Apparatus for deoxygenation and stirring **[74]**.

2.4 PREPARATION OF *CIS-SYN* AND *TRANS-ANTI* THYMINE DIMER

In order to identify thymine dimer and for quantification purposes, large quantities of *cis-syn* thymine dimer were required. Previous members of this research group have discussed the method of preparation of this dimer **[74]**. A brief description of the method is given here. The method is an adaptation of that developed by Beukers and Berends **[18]**. It involves the irradiation of a frozen thymine solution. Dimerisation via this route produces only the *cis-syn* stereoisomer of thymine dimer **[202]** and this arises mostly from singlet precursors formed in the frozen aggregates **[34]**. The quantum yield of dimer formation in ice (0.8) is much higher than that for irradiation of aqueous solutions **[203, 204]**. Therefore, high concentrations of the dimer can be obtained in this way. The frozen state of thymine is similar to the crystalline structure in the solid state. Water of crystallisation boosts the stability of the thymine crystalline structure; this in turn determines the quantum yield and the stereochemistry of thymine dimer **[202]**.

A Phillips HP-T 400 W medium pressure mercury vapour lamp was used for the thymine dimer preparation. Its protective pyrex cover was removed to enable irradiation with a wavelength of 265 nm, a necessity for direct thymine dimerisation. A protective aluminium guard was fitted over the back of the lamp to give the lamp support and provide protection from violent failure. It was then fitted in a black wooden box containing an aluminium tray at the base (See Figure 2.8). Finely crushed dry ice was placed onto the tray. A second slightly smaller tray was placed on top of the dry ice and a 2×10^{-2} M thymine solution was sprayed onto the top tray until an approximately 1-mm thick layer of frozen thymine solution was formed. The frozen solution was then irradiated for three 5-minute intervals with 5 minutes between each irradiation to prevent the heat generated by the lamp from thawing the frozen layer, i.e. a total of 30 minutes from the time of switching on the lamp. A volume of 500 mL of thymine solution was irradiated by this procedure. After each cycle, the irradiated film of thymine was removed, transferred into a beaker and thawed.

The contents of the beaker were heated to dissolve the precipitate and filtered hot to remove extraneous matter. The filtrate was then concentrated by evaporating it to a volume of about 150 mL and allowed to cool so as to cause precipitation of the dimer which was removed by filtration. This is possible because the solubilities of thymine photoproducts (dimers) and thymine in water at room temperature are 0.5 g L^{-1} and 4.0 g L^{-1}, respectively **[205]**. Thymine is considerably more soluble in ethanol than are the dimers. Hence the residue was washed with 30 mL portions of ethanol four times. After a further wash with water, the solid was dissolved in about 60 mL of water at 80 °C and then recrystallised. This process was done twice and finally the crystals dried in an oven at 40 °C for one hour giving a typical yield of about 10%. This purification procedure was necessary because small amounts of thymine monomer are known to coprecipitate with the dimer.

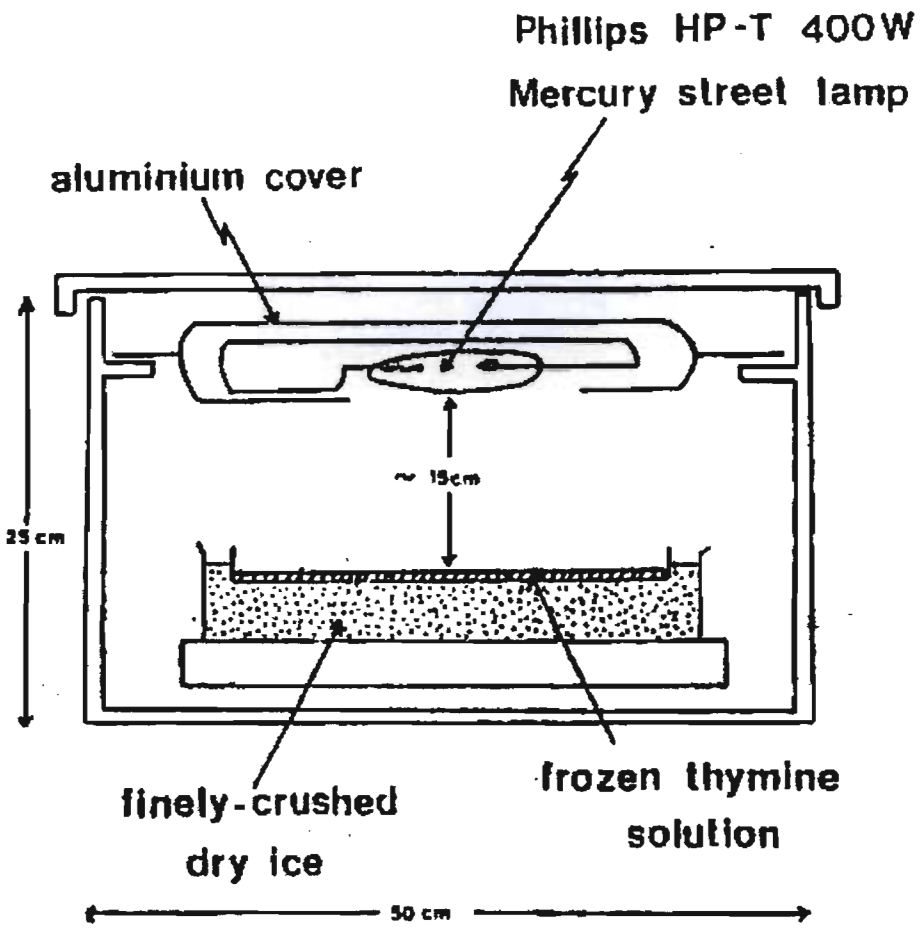

Figure 2.8: Apparatus used for irradiation of frozen thymine solutions to prepare *cis-syn* thymine dimer **[74]**.

The purity of the dimer formed was determined by UV absorption measurements. Exactly 0.0630 g of the prepared thymine dimer was weighed and dissolved in water in a 250 mL volumetric flask to give an assumed thymine dimer concentration of 9.992 x 10^{-4} M. Serial dilutions were made from this stock solution. The absorbance of the stock solution and the other solutions were measured at 235 nm and 265 nm against water as a blank in 1-cm pathlength quartz cuvette. From knowledge of the molar absorption coefficient of *cis-syn* thymine dimer, namely 1.5 x 10^3 M^{-1} cm^{-1} and 87 M^{-1} cm^{-1} for 235 nm and 265 nm, respectively **[35]**, exact concentrations were calculated and averaged. The purity was found to be greater than 99%. Using Waters' Millennium software for HPLC analysis, a further purity test was done. The slight difference in spectra for the same compound attributed to non-absorbance properties is referred to as the Spectral Contrast angle. The threshold angle is the maximum Spectral Contrast angle. If the former is greater than the latter, it implies that the spectrum (peak) is impure. The difference is calculated in terms of threshold purity **[206]**. Using this method, the purity for dimer was found to be 98.8%. The difference in the percentage between the two methods could be attributed to a small portion of the dimer reverting to the monomer in solution.

Cis-syn thymine dimer solutions were prepared as required since with time they are unstable and slowly revert to the monomer **[35]**. The UV spectrum of a dilute solution of the dimer prepared is shown in Figure 2.9 to show the lack of thymine monomer absorbance at 265 nm.

Together with *cis-syn* thymine dimer, *trans-anti* dimer had to be prepared for use as a standard for peak identification purposes on the HPLC. *Trans-anti* thymine dimer was prepared according to a modified method of Von Wilucki **[207]**. This method involves acetone-photosensitisation of thymine solution using a mercury lamp. In this process, all the four dimers of thymine are formed. However, *trans-anti* dimer has the lowest solubility **[207]** and precipitates out of solution leaving the other three dimer stereoisomers in solution.

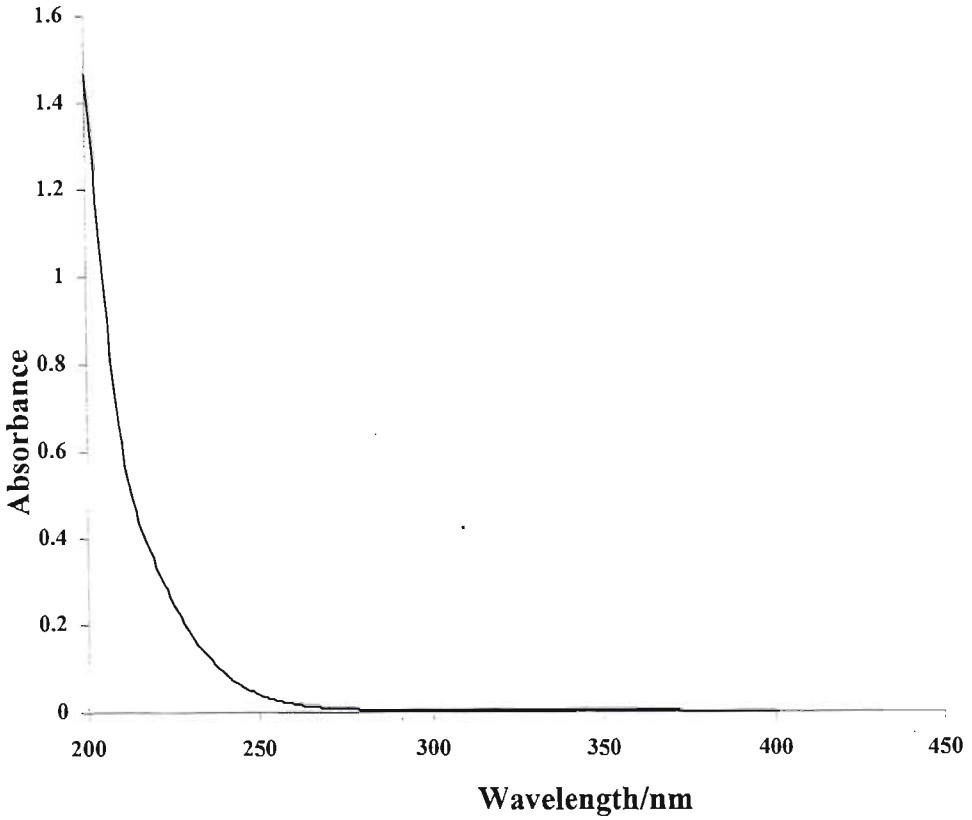

Figure 2.9: UV absorbance spectrum of an aqueous solution of *cis–syn* thymine dimer recorded in a 10 mm pathlength quartz cuvette.

A solution of thymine of concentration 2.8 x 10^{-2} M in acetone-water 80:20% by volume was irradiated for 150 hours with a Hanovia 125 W medium pressure mercury lamp (Figure 2.10). The lamp is surrounded by a water-cooled pyrex sleeve to ensure photosensitised irradiation of the solution. Thus the photoreaction proceeds via the desired pathway (i.e. $\lambda > 300$ nm). This avoids direct dimer formation. A water-jacket was placed around the photochemical reactor vessel for cooling and to prevent evaporation of the solvent and thus minimise coprecipitation of the unreacted thymine monomer. The photoreactor has a large capacity of 500 mL. Some thymine monomer could have precipitated together with the dimer. To remove any trace of monomer that might have formed, the precipitate was filtered, washed with absolute ethanol and then washed with water and dried in the oven at about 50 ^{o}C and left to cool in a desiccator. A typical yield of about 8% was found for

this dimer. Its purity was then determined by HPLC analysis. The threshold purity (described on page 63) from HPLC analysis by means of the Water's Millennium software showed a purity of greater than 97%.

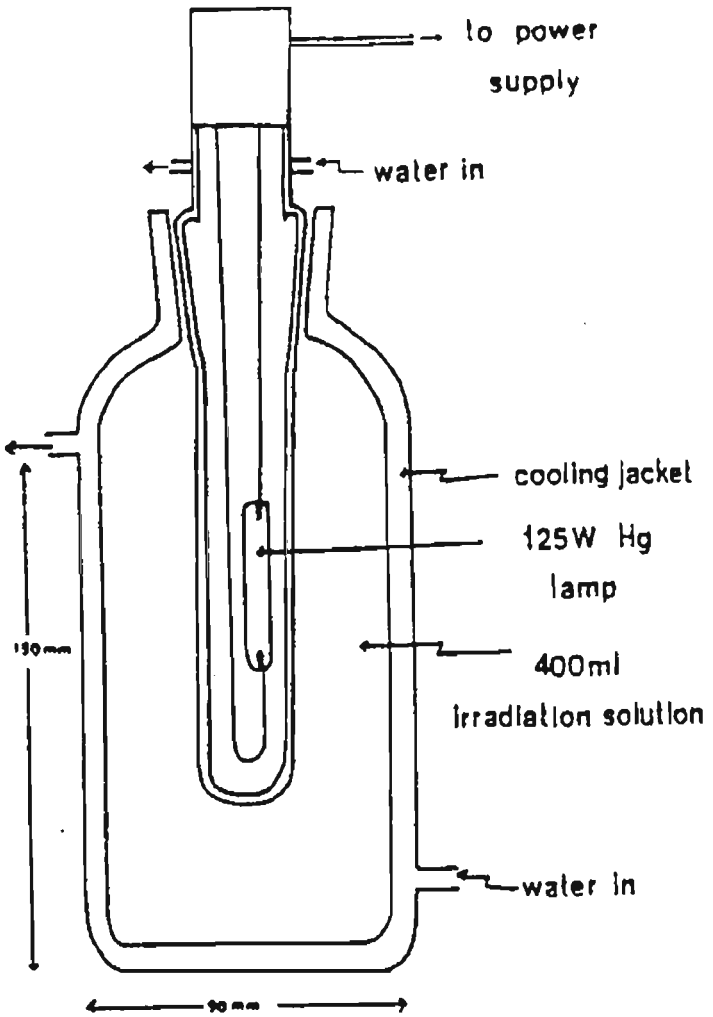

Fig 2.10: The Hanovia photochemical reactor used for *trans-anti* thymine dimer preparation **[74]**.

2.5 CHARACTERISATION OF THYMINE MONOMER AND THYMINE DIMER

In addition to characterisation by UV spectroscopy, *cis-syn* thymine dimer was also characterised by infrared spectroscopy (IR). An IR spectrum was obtained in a KBr matrix with a Nicolet Impact 410 spectrophotometer. The software for this spectrophotometer was Omnic FT-IR. IR spectroscopy cannot be employed

to distinguish the fine details in the difference between the isomers of the dimer. Hence the IR spectrum for *trans-anti* thymine dimer is indistinguishable from that of the *cis-syn* dimer. The IR spectrum of *cis-syn* thymine dimer is shown in Figure 2.11 with the following assignments: 1693 cm^{-1} (NHCONH); 1738 cm^{-1}, (CONHCO); 3224 cm^{-1}, (CONH). This compares very well with what Weinblum *et al.* **[208]** and Blackburn *et al.* **[209]** found, i.e. 1690 cm^{-1} (NHCONH); 1730 cm^{-1} and 1760 cm^{-1}, (CONHCO); 3210 cm^{-1} and 3300 cm^{-1}, (CONH). The small peak at 869 cm^{-1} is consistent with previous studies indicating that a peak at around 870 cm^{-1} is typical of thymine photoproducts **[205]** whereas a peak at around 957 cm^{-1} is characteristic of the cyclobutane ring **[210]**. These two peaks are absent from the IR spectrum of thymine monomer that is shown in Figure 2.12. The melting point of thymine monomer after recrystallisation was determined and found to be 316 °C. The melting point of the dimer was determined but sublimed at around 319 °C. This agrees with literature values of 320 °C **[18, 205, 209, 210]**. The slight difference between the literature value and the experimental value could be due to the presence of the less than 1% of thymine monomer impurity.

2.6 SEPARATION AND IDENTIFICATION OF PHOTOPRODUCTS BY HPLC

High pressure liquid chromatography (HPLC) has been previously used for the separation and quantitation of nucleosides in urine and other biological fluids **[211]**. In particular, octadecyl silane (ODS) reverse-phase columns have been used to separate isomers of thymine dimer **[212, 213]**. HPLC has also seen wide application in vitamin analysis **[214]**. Both ODS and Nucleosil 100 C_{18} reversed-phase columns have previously been used in vitamin analysis **[215 - 217]**. It therefore seemed appropriate to use reversed-phase columns in the HPLC analysis to separate the vitamin and thymine photoproducts. Highly polar solutes elute from reversed-phase columns before less polar solutes. Low polarity solutes are retained due to their affinity for the low polarity stationary phase relative to the more polar mobile phase.

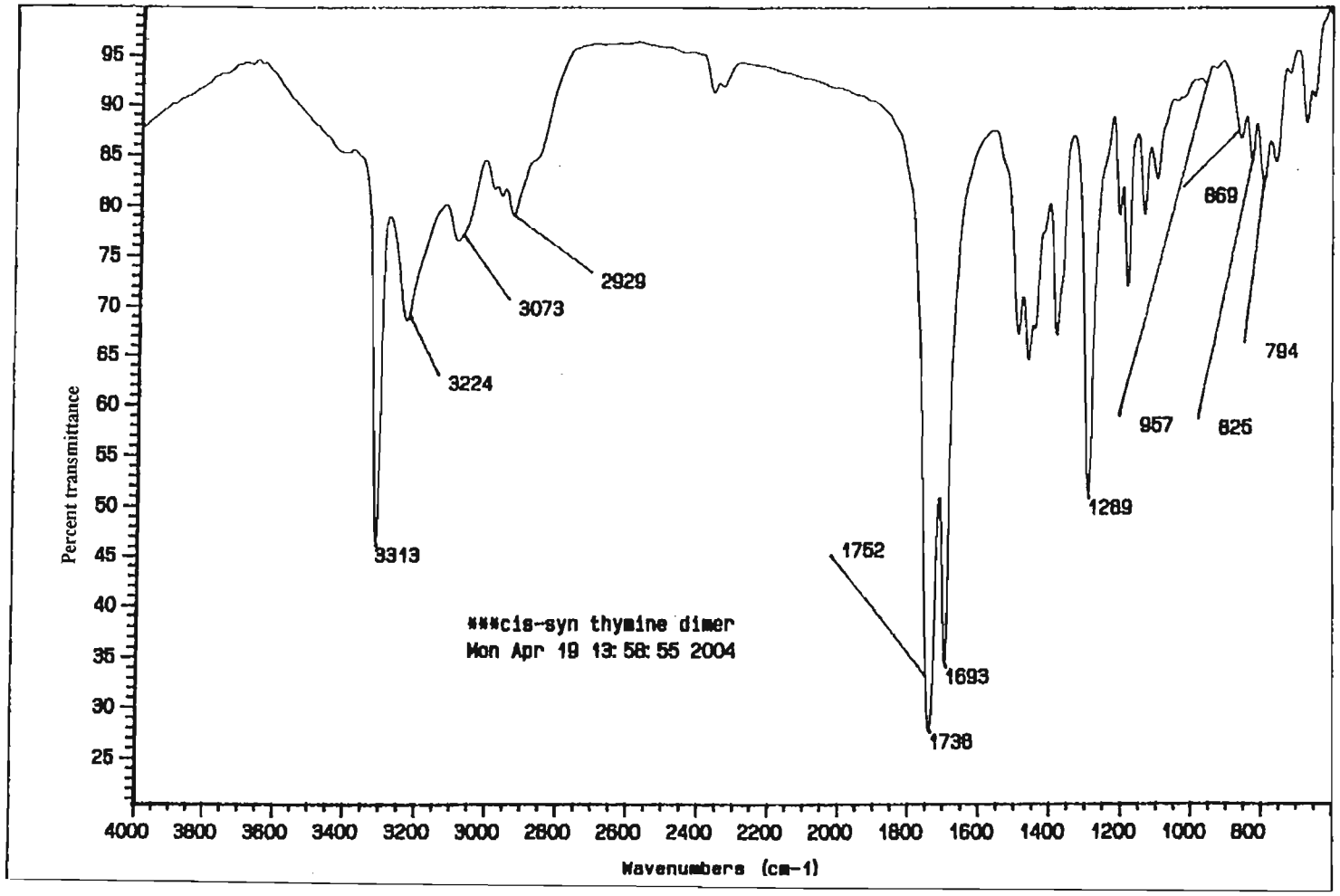

Figure 2.11: IR spectrum of *cis-syn* thymine dimer (KBr disc).

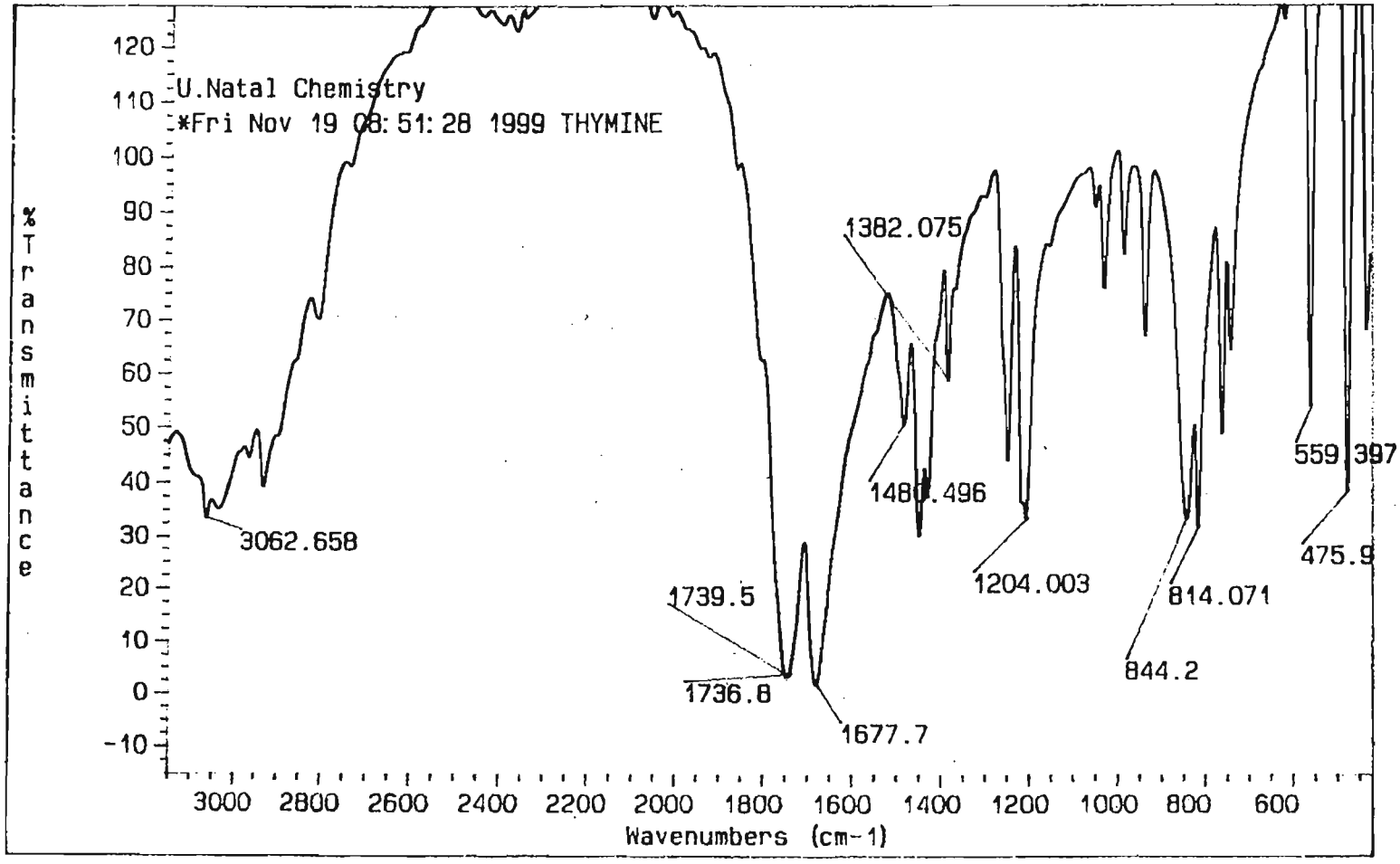

Figure 2.12: IR spectrum of thymine monomer (KBr disc).

2.6.1 HPLC EQUIPMENT AND OPERATION

A Waters 600 multi-solvent delivery system with 225 µl pump heads was used in these studies. The fact that these 225 µl pump heads are larger than the standard 100 µl pump heads makes the system suitable for both semi-preparative and analytical work. Only analytical work was performed here. Previous members of the research group have discussed the operation of the multi-solvent delivery system **[56, 218]**, so it suffices to give a brief description here. The multi-solvent delivery system has both isocratic and gradient features. The isocratic mode is most suitable for sample components that are chemically similar. This was the mode that was used to separate the photoproducts in this work. The gradient feature allows the separation of complex mixtures and the performance of clean-up procedures. During the early part of this work, U6K and Rheodyne injectors were used. For these injectors, a Hamilton airtight 100 µl syringe that has a precision of 0.65% (as per the specification of the manufacturer) was used to introduce a sample into the variable loop of the U6K injector. Later on, a plastic hypodermic injection syringe was used for the Rheodyne injector port whose loop was not variable, i.e. 100 µl. After acquisition of the Perkin-Elmer 2000 series auto-sampler the injections were automated and consequently the precision improved and the instability of the baseline was greatly reduced. The U6K injector is designed to allow injections at a system pressure of up to 6000 psi without solvent flow interruption. It can accommodate analytical and preparative injections from a few microlitres to 2 millilitres without making any changes to the injector. The 7010 Rheodyne sample injection valve had a Rheodyne 7012 loop filler port with a capacity of 100 µl connected to it. The loop filler port permits loading of the sample by means of an injection syringe. It can also load the sample by suction from a vial. A hypodermic injection syringe was used when the Rheodyne 7010 injector was fitted. The Perkin-Elmer auto-sampler has a dual syringe system that contains the sample syringe and the flush syringe. The sample syringe picks up the sample by suction. Volumes as low as 5 µl can be picked up with high precision. The autosampler allows both dilution and derivatisation of samples before injection. The sample syringe could go to a

minimum level in the vial and below this level it could not collect any sample. After two injections of 100 µl each, the 480 µl irradiated sample went below the minimum level. Hence a maximum of only two repeat injections could be done from a given irradiated sample. The flushing process cleans the syringe using methanol or the mobile phase and thus minimises carry-over into the column.

During the initial part of the studies, we used a Waters 990 photodiode array detector and an NEC, APCII personal computer for data acquisition and peak area integration. Later on, another model (Waters 996 photodiode array detector) was acquired. The photodiode array detector (PDA) consists of 512 photodiodes with a range from 190 nm to 800 nm with an optical resolution of 1.2 nm per diode. The system thus allows scans over the ultraviolet and visible regions. In this way large amounts of chromatographic and spectral data may be acquired from a single analysis and stored on the computer. Such data can be used to generate a 3-D chromatogram (absorbance versus wavelength versus time). A De'Mark Pentium II personal computer with Waters Millennium version 3.2 software was used to operate and collect data from the PDA. In the course of the studies it was upgraded to version 4.0. The software has the facility to determine purity of the peaks, their height, percentage areas of the different peaks relative to the total peak area on a chromatogram, and many other parameters. These help in sample analysis and peak identification. The PDA was allowed to warm up for one hour before use so as to attain maximum performance parameters.

To avoid introduction of particulate matter into the HPLC system, to eliminate contamination during analysis and to prolong the lifetime of the columns, some precautionary measures were taken. HPLC-grade solvents and water purified through a Millipore Milli-Q^{50} water purification system were used to make up the mobile phase. They were always filtered through Millipore 0.45 µm HV organic aqueous compatible filters. For this work the mobile phase consisted of various compositions of methanol and water. The sum of the individual volumes of water and methanol is always greater than the volume of their mixture. The difference in volume is normally referred to as the negative

volume. This phenomenon is due to hydrogen bonding present in the mixture. Consequently air bubbles are formed when the two solvents are mixed at low pressures. Bubbles are undesirable in HPLC since they increase the signal to noise ratio and cause flow irregularity. The low pressure-mixing chamber of the pump is therefore not very suitable for mixing volumes of water and methanol. For this reason, water and methanol were always pre-mixed, filtered and allowed to stand for at least three minutes before being introduced into the delivery system. This worked well for isocratic modes of elution. In-chamber mixing of solvents could not be avoided while using the instrument on gradient mode. To minimise the effect of bubbles further, the system had to be primed every morning and sparged with high purity helium at a flow rate of 30 mL min^{-1} during runs. Helium is virtually insoluble in solvents and displaces any dissolved gas **[219]**. A disposable Waters Guard-Pak μ-Bondapak C_{18} precolumn insert was included between the solvent delivery system and the column. This helps to filter particulate matter and other impurities and prolong the life of the column. A loss of 2 to 10% in resolution is incurred when using a guard column **[219]** but the loss was acceptable since the resolution was adequate with the guard column. Irradiated samples were filtered before injection through a 0.45 μm Millipore Millex-HV solvent resistant syringe filter unit. After use the mobile phase was passed through the column and a blank injection made to make sure that the column was clean. It was then stored in methanol. After storage for long period of time, five column volumes of the eluent were always passed through the column before use. This was to ensure equilibration and thus minimise changes in retention times. It also has the advantage of prolonging the life of the column. To protect the pump head from damage and fast deterioration, flow rates were always increased or decreased in steps of 0.2 mL min^{-1}. This also has the advantage of protecting the column packing since sudden rises or falls in pressure in the column can damage it.

Some precautionary measures were taken to ensure that there was no carry-over in the injections. This involved the syringe, the injection port and the column. The Hamilton syringe was thoroughly rinsed with methanol followed by rinsing with the mobile phase before use. Once every fortnight, it was cleaned with methanol in an ultrasonic bath. The loop filler ports of the U6K and

Rheodyne injectors were flushed with methanol every week to clear the injector of any particulate matter that might have accumulated. After every run a blank consisting of the mobile phase was always injected to make sure that the column was clean and that no solute had been retained. With the acquisition of the Perkin-Elmer autosampler, cleaning of the syringe and the injection port was automated. Twenty flush cycles between runs were done between injections using methanol. The autosampler was first flushed with 2-propanol whenever it had not been in use for at least 24 hours and then flushed with methanol. All these precautions ensured that all the chromatograms and spectra obtained originated from the sample being analysed and not from any other source.

2.6.2 HPLC TERMINOLOGY

Reversed phase ODS and Nucleosil columns have wide applications in separation and quantification of thymine photoproducts and vitamins. They work quite well with polar mobile phases like water and methanol, which are cheaper than other solvents. Ultracarb ODS, Spherisorb ODS and Nucleosil 100 C_{18} columns were thus chosen and tested for their suitability in our investigation.

A number of terms are used to compare the characteristics of HPLC columns. These include capacity factor (k′), selectivity (α), resolution (R) and number of theoretical plates (N) - a measure of efficiency. The capacity factor, k′ is a measure of the ability of a particular column to retain a specific component. It is used frequently to locate or identify a peak. For a given component, k′ is defined by the equation:

$$k' = \frac{V_1 - V_0}{V_0} \qquad 2.1$$

where V_1 is the retention volume of the component (the volume required to elute the component) and V_0 is the void volume, the volume required to elute an unretained compound. The void volume of a system in terms of volume is a

measure of the system's volume from the injector to the detector. It includes the volume inside as well as outside the packing particles plus the tubing. The capacity factor is a function of solvent polarity and the nature of the stationary phase. The value is unaffected by variables such as flow rate and column dimensions. The optimum value of k′ is between 2 and 6 **[218]**.

The selectivity factor or the separation factor, α, sometimes referred to as the chemistry factor of a column, is a measure of the ability to separate a component compound of a sample. Selectivity is defined as in Equation 2.2 and expresses the relative positions of two peaks. It gives no indication of the symmetry and geometry of the peak.

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0} = \frac{k_2'}{k_1'} \qquad 2.2$$

where V_1 is the retention volume of peak 1, V_2 is the retention volume of peak 2 and V_0 is the void volume. It is conventional to place the larger k′ value in the numerator and the other in the denominator so that $\alpha \geq 1.0$. If two compounds elute with $\alpha = 1.0$, it implies that their k′ values are identical and a single peak is thus observed. For satisfactory separation of components, α should be greater than 1.5 **[218]**. This parameter depends on the polarity of the components. It does not take into account the peak width. Figure 2.13 shows parameters used in calculating selectivity, capacity factor and resolution. Where V_0, V_1, V_2, W_1 and W_2 in the figure represent the void volume of the column, the retention volumes of components 1 and 2 and the width of their peaks respectively.

The efficiency of a column is a measure of the degree of sharpness of the peaks or the deviation of a band around the centre. It is a measure of band spreading on an ongoing basis. A number of factors contribute to spreading of the band, for example, end fittings, detector cell, particle size, mobile phase, flow rate, *etc.* It is measured in terms of the number of theoretical plates (N). Various methods can be used to calculate N. The most appropriate formula for its calculation is the $N_{5\sigma}$ formula (Equation 2.3) that accounts for peak tailing.

$$N_{5\sigma} = 25\left(\frac{V}{W}\right)^2 \qquad 2.3$$

where V is the retention volume of the peak and W is the width of the peak at 4.4% of peak height, h, is defined as seen in the schematic diagram (Figure 2.14).

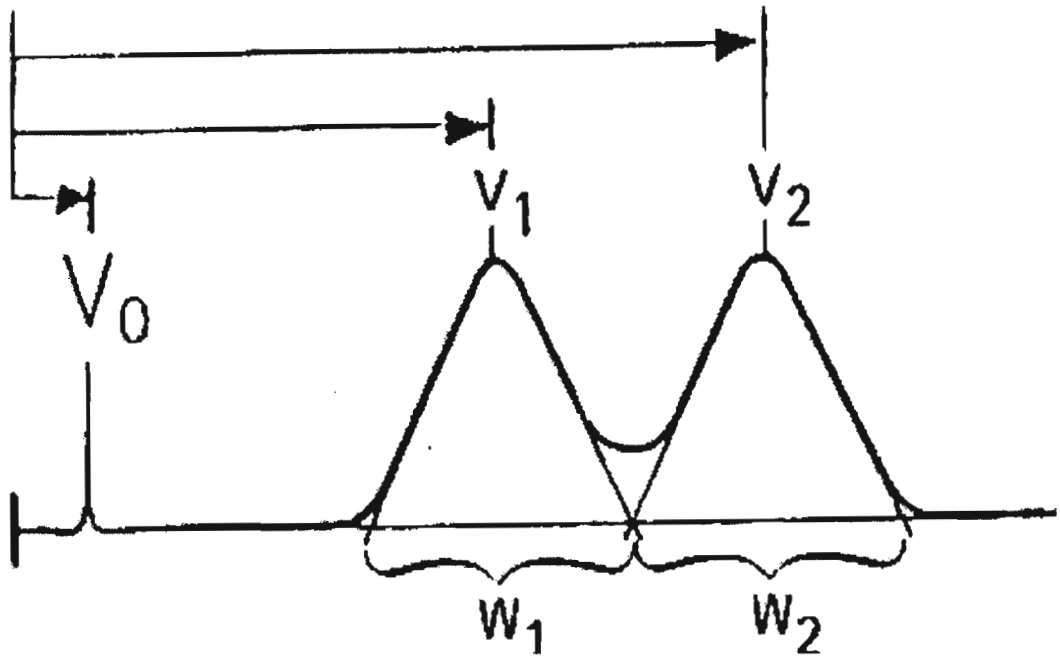

Figure 2.13: A schematic chromatogram showing the void volume of a column, the volumes and base widths of peaks that were used in the calculation of capacity factor, k′; selectivity, α and resolution, R **[74]**.

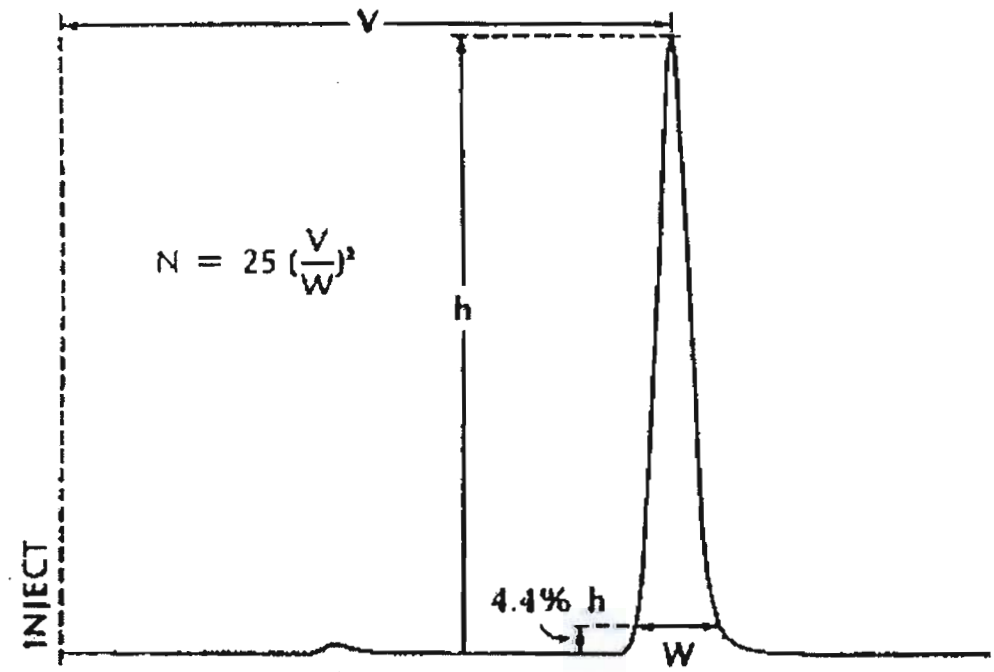

Figure 2.14: Diagram showing the parameters used to calculate the number of theoretical plates, N, by the $N_{5\sigma}$ method **[74]**.

This method is often referred to as the 5-sigma method. It is the most accurate method for the calculation of column efficiency. It was the method used in this work. The plate count will not be the same for systems where the solvents, instrument, flow rate, column or other variables are changed. The larger the value, the greater the efficiency.

Resolution (R) is a measure of the separation of two peaks and takes into account the width of the peak (or band spreading) and is defined in Equation 2.4. Resolution is the ultimate goal of chromatography. Higher values may be required for some liquid chromatography (LC) separation applications whereas low values may be required for others **[220]**.

$$R = 2\left(\frac{V_2 - V_1}{W_1 - W_2}\right) \qquad 2.4$$

If the capacity factor, selectivity and plate count are combined, the resolution can be calculated as given in Equation 2.5.

$$R = \frac{(\alpha - 1)k'\sqrt{N}}{4\alpha(k' + 1)} \qquad 2.5$$

For practical purposes, to improve R we manipulate k′ and α. As with k′, an initial increase in α increases R dramatically. This effect is less significant at higher values where k′ approaches unity. For good resolution between peaks the value of R should be greater than 1.5 **[221]**. The effect of N on resolution implies that in order to double R, N must be increased by a factor of four.

2.6.3 COLUMN TESTING PROCEDURE

Three columns were tested and compared for five parameters, namely tailing, theoretical plates, capacity factor, selectivity and resolution. While comparing these parameters, the flow rate and composition of the mobile phase were considered. The values for tailing for the 5 ODS (20) column were generated

by the System Suitability software provided courtesy of Microsep Limited, Durban, South Africa. All the three columns were made of stainless steel and were of 250 mm length. The columns were packed with material bonded to silica gel as the base. The length of the alkyl chain bonded to the silica contained 18 carbons. The size of the particles in the columns was 5 μm with the residual silanols capped except for the Nucleosil 100 C_{18}, which did not have residual silanols. The names and technical specifications for these columns showing their lengths, internal diameters and the weight percent of carbon atoms bonded to the silica measured by elemental analysis, i.e. carbon loading are given in Table 2.1.

Table 2.1: Specifications of the three HPLC columns tested.

Column	Dimensions of columns/mm	Carbon loading/%
Ultracarb 5ODS (20)	250 x 10	12
Spherisorb 5ODS (2)	250 x 10	22
Nucleosil 100 C_{18}	250 x 4.60	14

A brief description of the column testing method is given here. All the five parameters except the number of theoretical plates require the void volume of the column to be measured. The void volume can be experimentally determined by using an analyte that is not retained on the column and does not react with it. Uracil and theophylline are the common markers used to determine void volumes for reversed-phase columns **[222]**. Void volumes for columns used in this work were calculated from the retention time of uracil, as a marker for t_o, the retention time that gives the void volume, V_o. The void volume was arrived at by multiplying t_o with the flow rate. This gave the total void volume of the columns. The void volume could also be calculated by making use of Equation 2.6 and the approximate pore volumes of the columns provided by the manufacturers. To find the void volume associated with the tubing, the column was disconnected and the volume determined by injection of uracil.

$$V_0 = \frac{(ID)^2 \pi P_v L}{4} + V_t \qquad 2.6$$

where P_v is the pore volume in mL g^{-1}, ID is the internal diameter of the column, L is the length in cm of the column and V_t, is the volume of the tubing. The calculated and experimentally determined results are shown in Table 2.2.

Table 2.2: Volumes in mL for calculated, experimental and total void volumes used in calculating HPLC parameters for the columns tested.

Column or tubing	Experimental V_o/mL	Calculated V_o/mL	Total V_o/ mL used
5 ODS (2)	9.81	10.11	10.6
5 ODS (20)	14.73	14.69	15.53 and 15.68
Nucleosil 100 C_{18}	2.5	2.75*	3.3
Tubing without autosamlper	0.80	None	-
Tubing with autosamlper	0.95	None	-

(*) was given by the manufacturer **[222]**.

The test sample used to optimise the resolution conditions was an irradiated solution containing 1 x 10^{-2} M thymine and 1 x 10^{-3} M PABA. It was convenient to use the irradiated sample because it contained all the four isomers of thymine dimer and other photoproducts. The Osram HBO 500 W high pressure mercury lamp was used for the irradiations. The peaks in the chromatogram of irradiated sample were used to determine the capacity factor, selectivity, efficiency and resolution. The peaks considered were those of unidentified photoproducts, unreacted thymine, *cis-syn* thymine dimer and PABA. Thymine dimers were identified by injections of prepared *cis-syn* thymine dimer standards and their relative positions. These samples were

considered representative of the type of separation required at the concentrations produced in the photolysis experiments.

In the testing procedure, the flow rate, mobile phase composition and volume of sample injected were varied. Because of the prohibitive cost of acetonitrile, and based on the results of previous researchers in this laboratory **[56, 74]** that its mixture with water gave poor separation, we settled on water, methanol and methanol/water mixtures as mobile phases to obtain the columns' performance parameters and to optimise the resolution conditions. The water/methanol mixture gave significantly better resolution and will therefore be discussed in the following section. An injection volume of 100 μL was initially used to ensure satisfactory precision but this injection volume caused band tailing. A volume of 80 μL gave the best compromise between precision and band tailing. Lower injection volumes decreased absorbance and caused large errors in peak area and thus reduced precision considerably. For the semi-preparative columns flow rates ranging from 0.9 mL min^{-1} to 1.5 mL min^{-1} were used, whereas for the analytical column, flow rates ranging from 0.3 mL min^{-1} to 1.5 mL min^{-1} were tested.

2.6.4 RESULTS OF COLUMN TESTING

Figure 2.15 shows the change in the capacity factor of the Nucleosil 100 C_{18} column using thymine and PABA peaks as the content of methanol in the mobile phase is varied. It is evident that the capacity factor decreases with increase in methanol content of the mobile phase, i.e. an increase in polarity of the mobile phase reduces the retention times of different components.

Figure 2.16 shows the change in the capacity factor of the Nucleosil 100 C_{18} column as the flow rate of the mobile phase is varied. It is evident that there is an appreciable change in the capacity factor, k′, as the flow rate is varied. It decreases as the flow rate increases and tends to level off at high flow rates.

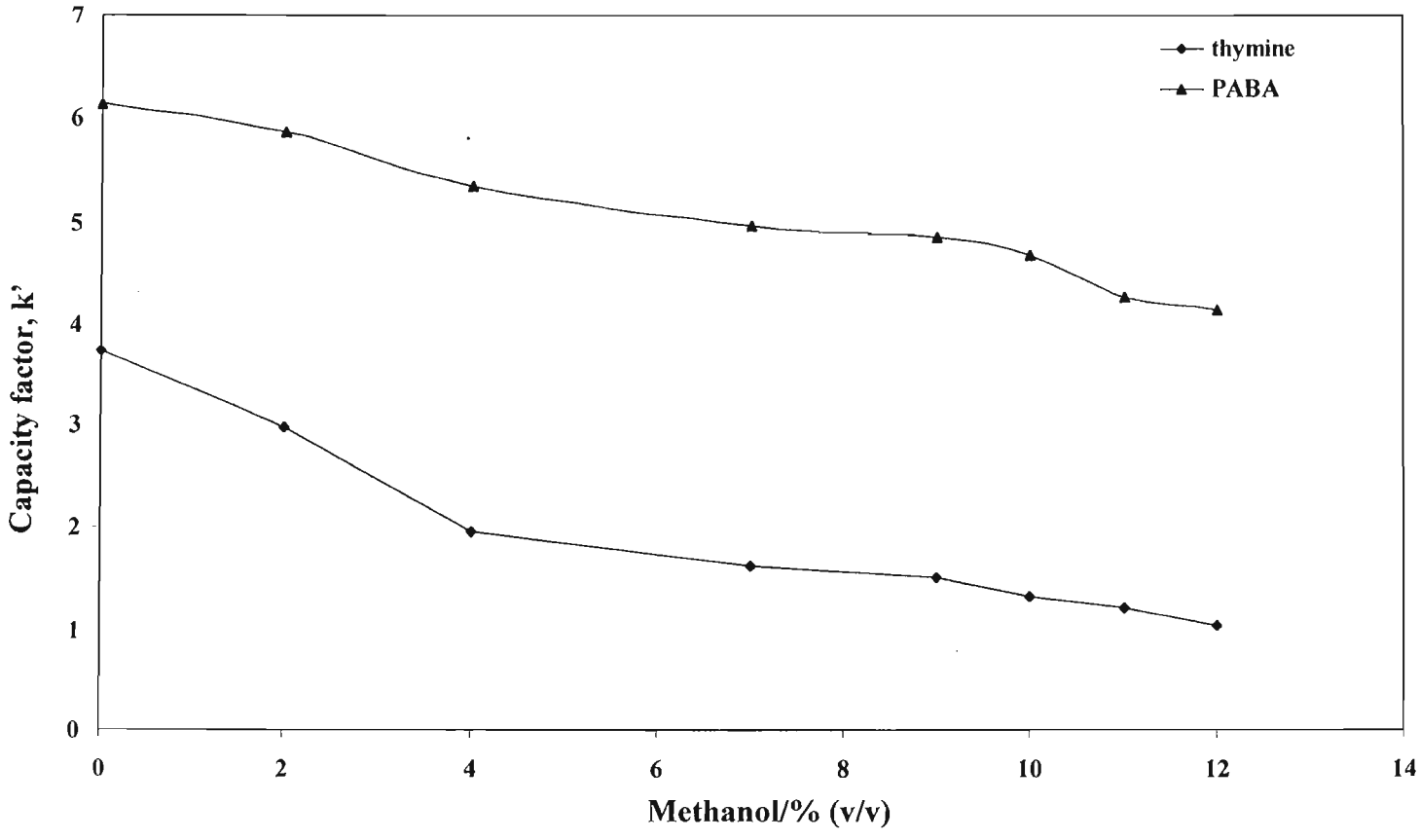

Figure 2.15: The effect of methanol content in the mobile phase on the capacity factor of the Nucleosil 100 C_{18} column at a flow rate of 1 mL min^{-1} using the thymine and PABA peaks monitored at 220 nm.

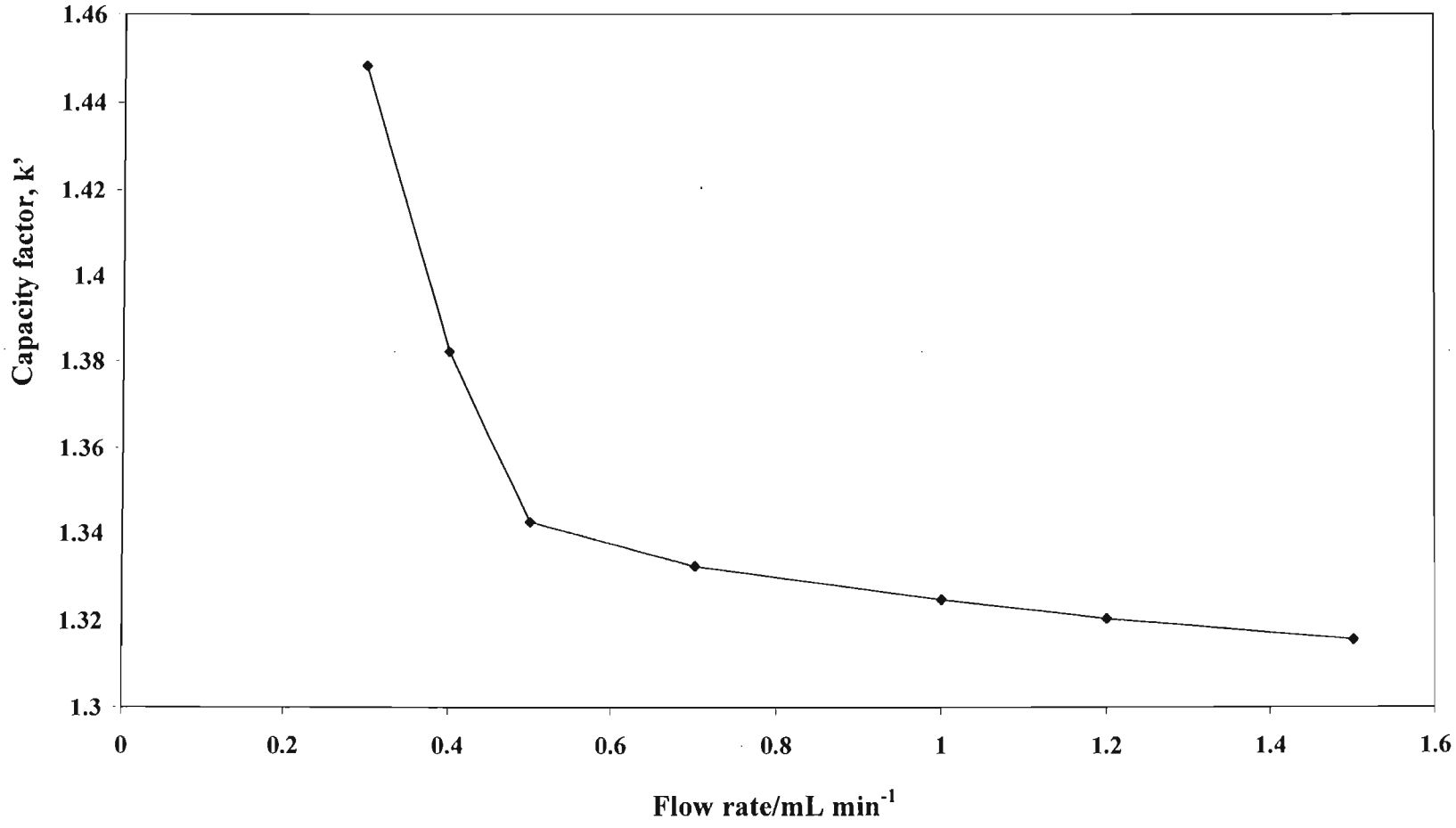

Figure 2.16: The effect of flow rate of the mobile phase on the capacity factor of the Nucleosil 100 C_{18} column for 10% (v/v) methanol in the mobile phase using the thymine peak monitored at 220 nm.

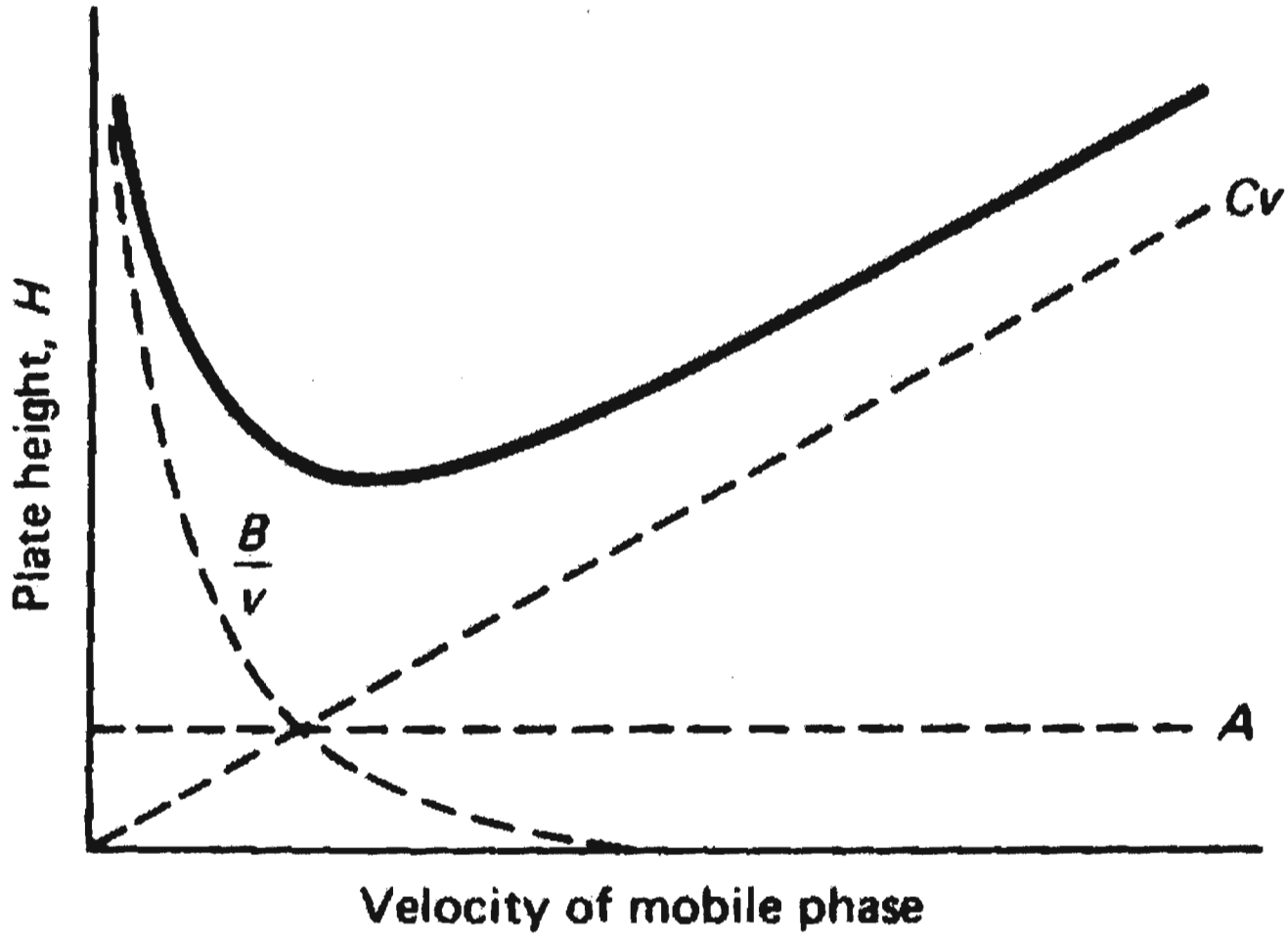

Figure 2.17: The effect of velocity of mobile phase on the efficiency of a column [223].

The Van Deemter equation shown in Equation 2.7 **[223]** explains column efficiency (H) (defined as plate height in Figure 2.17) in terms of factors that cause tailing and band broadening:

$$H = A + \frac{B}{V} + CV \qquad 2.7$$

where V is the flow velocity and A, B and C represent the amount of band broadening due to lack of uniform flow, longitudinal diffusion and resistance to mass transfer, respectively. This relationship is depicted graphically in Figure 2.17. The three factors represented in the equation are collectively referred to as the Poiseuille flow dispersion. This is the net effect of the three factors. The continuous line in Figure 2.17 represents this. Other factors that cause tailing and band broadening are the injector, the detector and connecting tubes and fittings. They have a pronounced effect when the flow passes through tubing that abruptly experiences changes in diameter.

By using the parameters discussed earlier, for instance peak width, the Waters System Suitability Software can generate the amount of tailing in the thymine peak. Tailing is measured in terms of a tailing factor, T, given by the mathematical expression below.

$$T = \frac{W_{0.5}}{2f}$$

where $W_{0.5}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak. The value of T is therefore dimensionless.

As can be seen in Figure 2.18, there is an appreciable increase in tailing of the thymine peak with an increase in the flow rate of the mobile phase. The tailing of the thymine peak gives an indication of tailing in other peaks. Nevertheless, as seen in Figure 2.19, tailing decreases with an increase in the amount of methanol in the eluent. This can be attributed to the increase in the ability of methanol to force the elution of the component and thus decreases tailing.

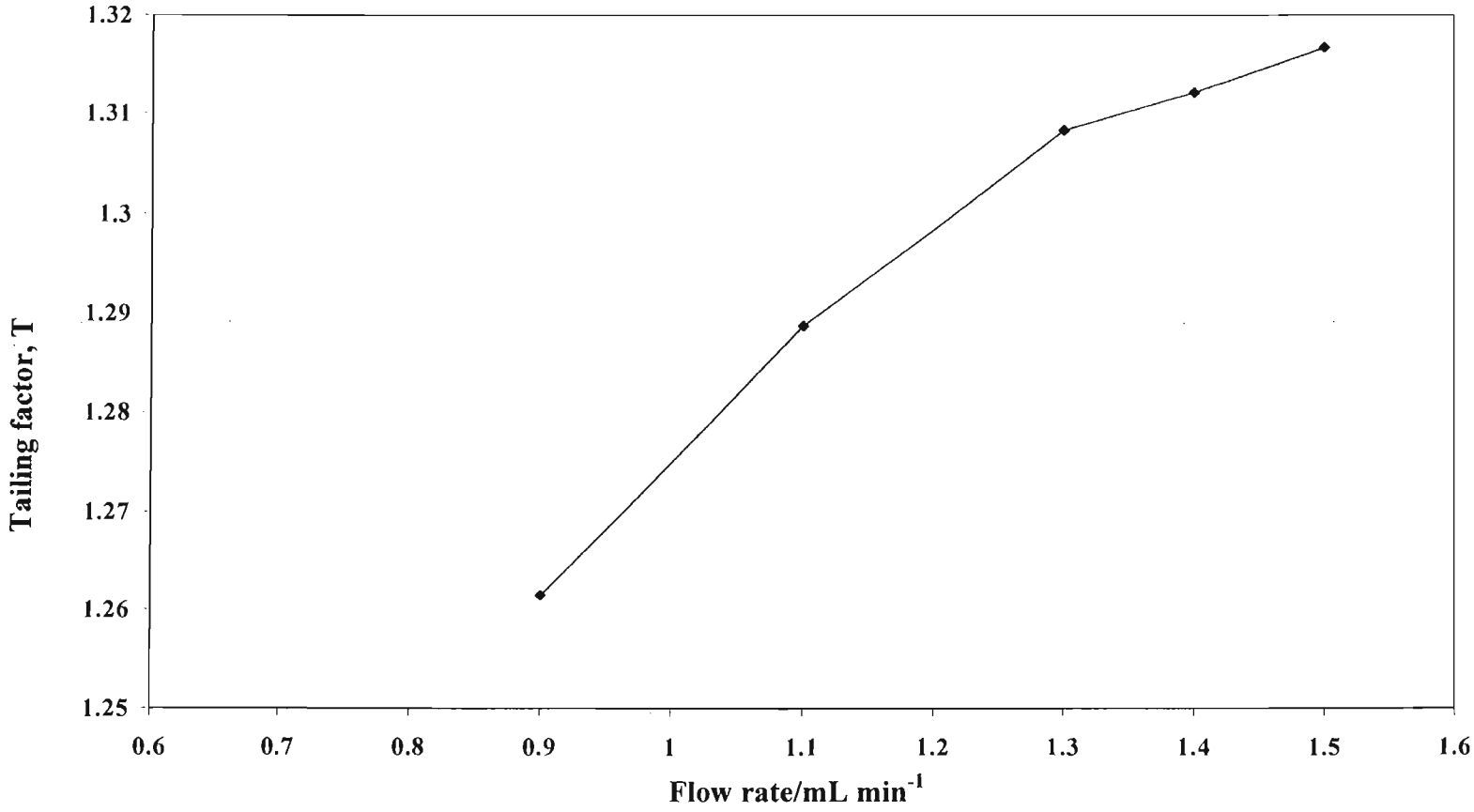

Figure 2.18: The effect of flow rate on tailing of the thymine peak for the 5 ODS (20) column with a 5 % (v/v) methanol-water mobile phase monitored at 220 nm.

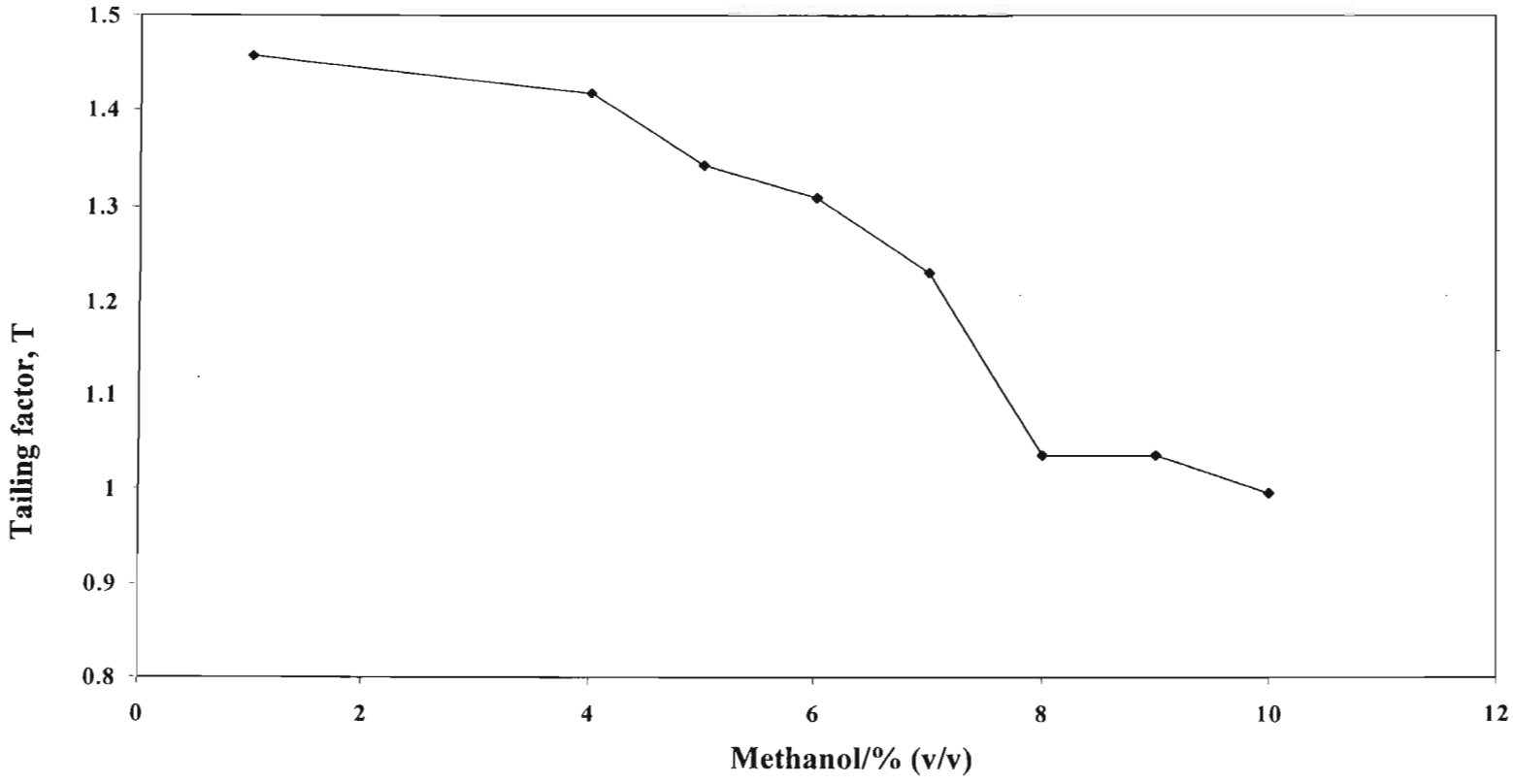

Figure 2.19: The effect of methanol content in the mobile phase at a flow rate of 1.5 mL min^{-1} on tailing of the thymine peak monitored at 220 nm for the Spherisorb 5 ODS (20) column.

Figure 2.20 shows a comparison of the selectivity for two columns when the amount of methanol in the mobile phase is varied. Both columns show a gradual increase in selectivity as the methanol content in the mobile phase is increased from 0% (pure water) to about 6%. Methanol content higher than 6% gives a rapid increase in the selectivity.

Results of the resolution tests for the Ultracarb 5 ODS (20) column are shown in Figure 2.21. Resolution of peaks increases with an increase in methanol content in the mobile phase up to a maximum of about 2%. There is no further increase in resolution as the percentage of methanol is increased. But after 8% (v/v) methanol the resolution decreases with further increase in methanol content. The resolution of peaks for the column was done at three different flow rates. It is evident from the figure that, the lower the flow rate, the higher the resolution. A flow of 1.0 mL min^{-1} had the highest resolution whereas a flow rate of 1.5 mL min^{-1} had the lowest resolution. The highest resolution for all the three flow rates is between 2% and 8% (v/v) methanol.

Figure 2.22 is a comparison of the resolution of all the three columns for a flow rate of 1.0 mL min^{-1}. It is evident that, of the three columns, the Ultracarb 5 ODS (20) provides the highest resolution when all the other parameters are the same. The maximum resolution for this column is obtained using about 5% (v/v) methanol. Therefore the Ultracarb 5 ODS (20) semi-preparative column is superior followed by the Nucleosil 100 C_{18} column and lastly the Spherisorb 5 ODS (2) column.

The efficiency of the Ultracarb 5 ODS (20) semi-preparative column was therefore tested at different flow rates. Efficiency in terms of theoretical plates for the column at four different flow rates is shown in Figure 2.23. Generally, the efficiency increases with increase in methanol content for low percentage values and decreases as the content of methanol increases. It is also evident from the plots that the higher the flow rate, the greater the efficiency for this column. The efficiency is highest when the flow rate is 1.5 mL min^{-1} and lowest when the flow rate is 1.0 mL min^{-1}.

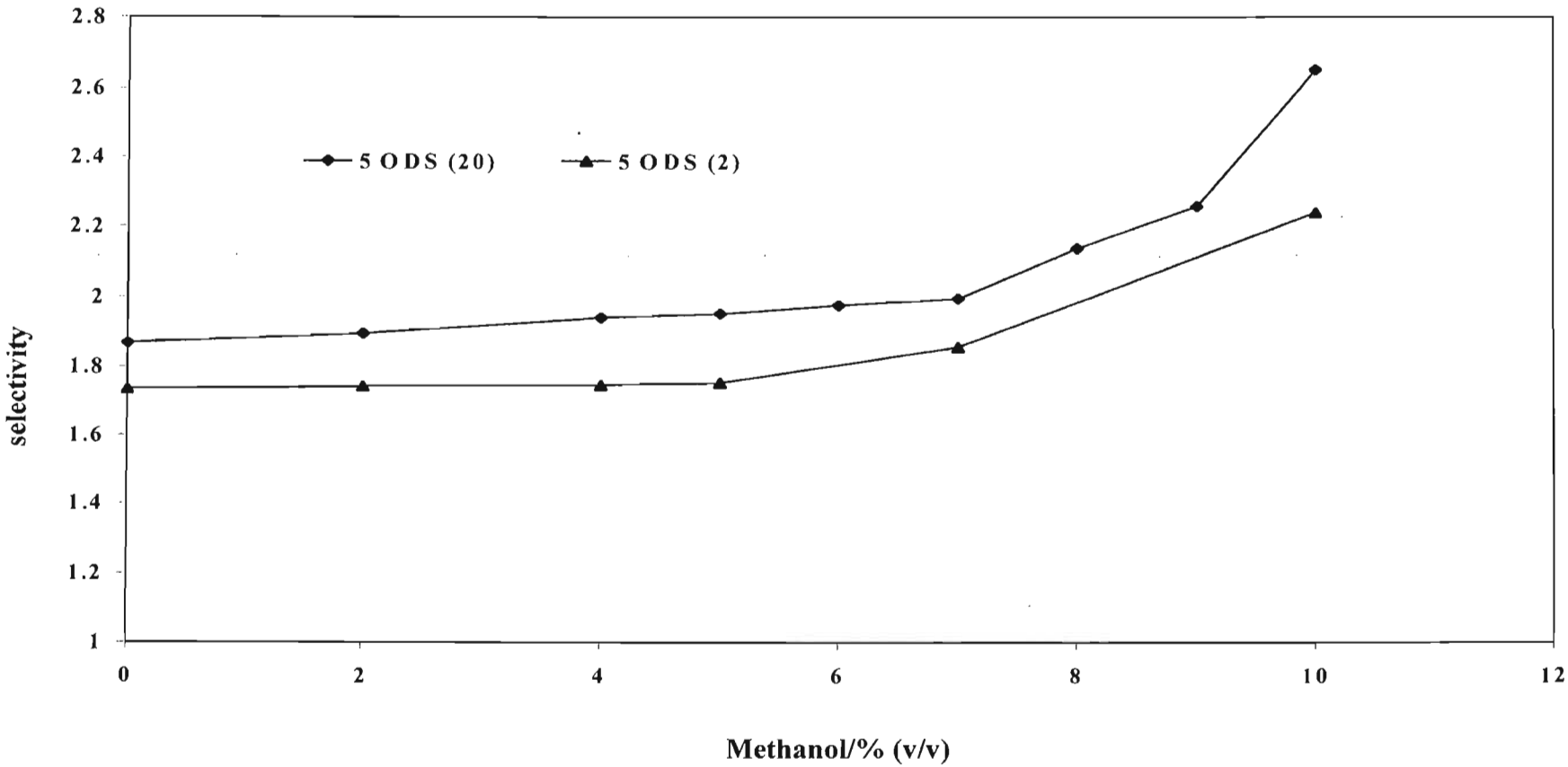

Figure 2.20: The effect of methanol content in the mobile phase on the selectivity of two different columns at a flow rate of 1.5 mL min^{-1} using a thymine peak and a peak of an unidentified photoproduct monitored at 220 nm.

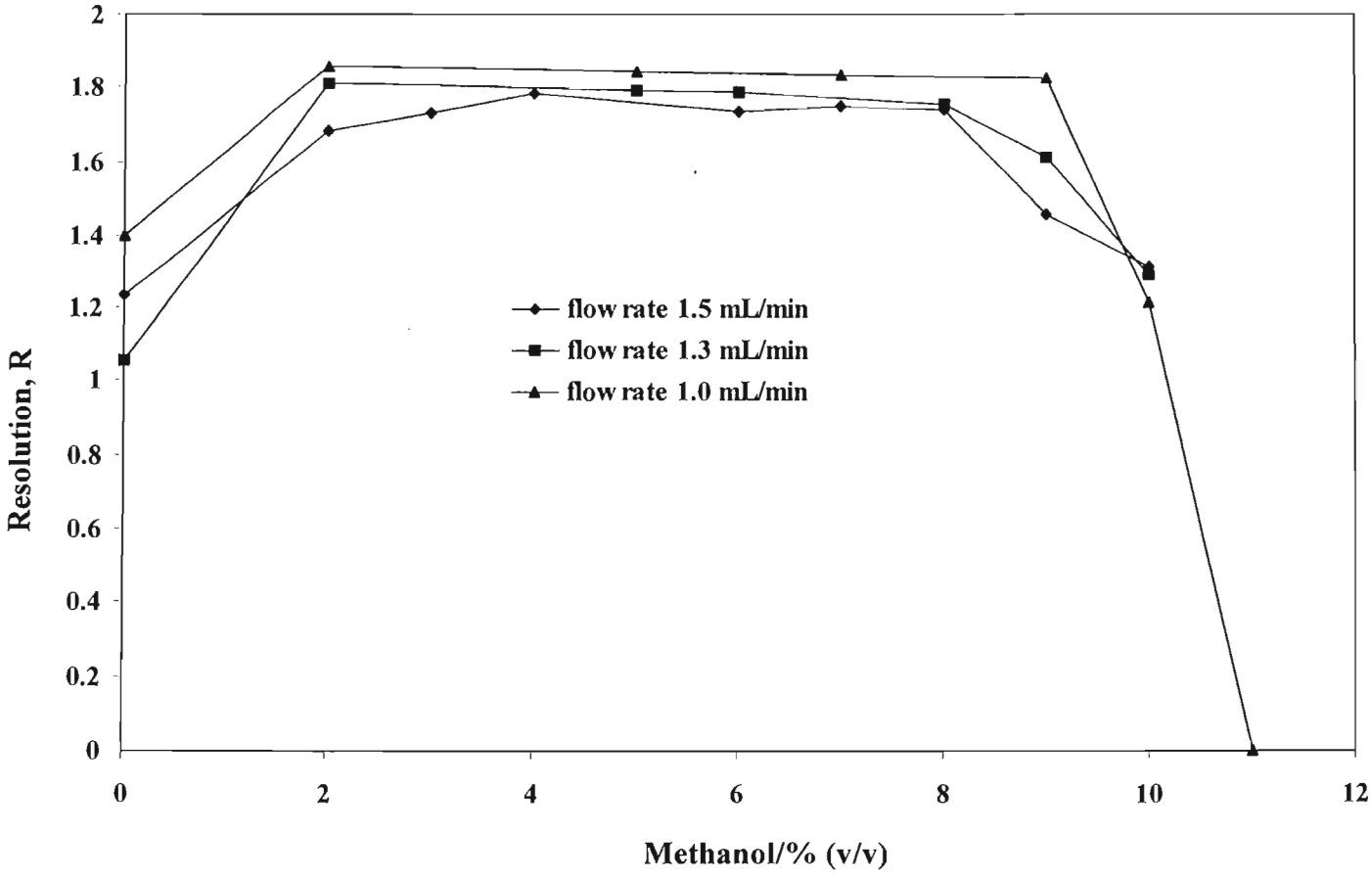

Figure 2.21: Effect of methanol content in the mobile phase on the resolution of the thymine peak and the *cis-syn* thymine dimer peak separated with the Ultracarb 5 ODS (20) column at different flow rates and monitored at 220 nm.

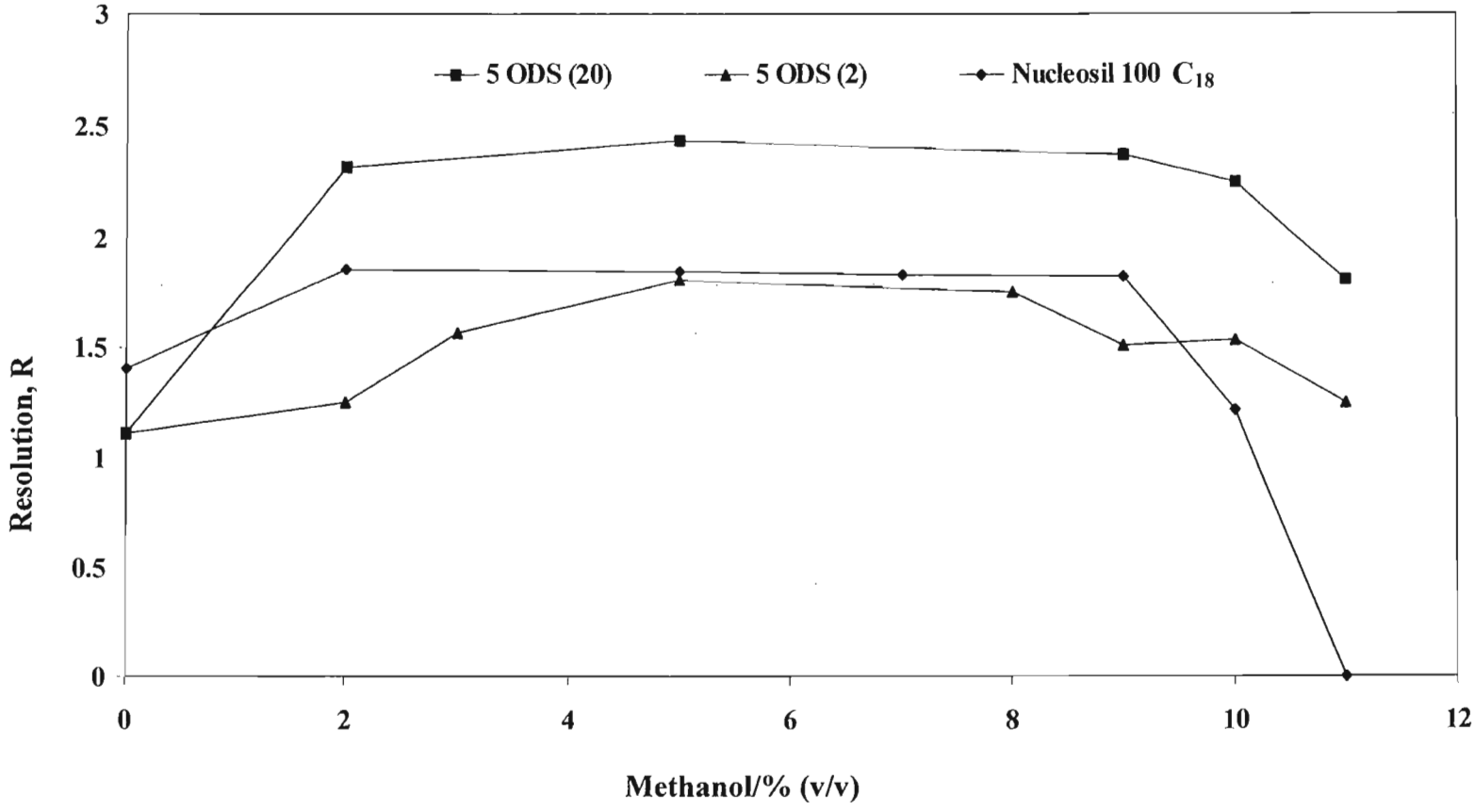

Figure 2.22: The effect of methanol content in the mobile phase on the resolution for the three columns tested. The mobile phase flow rate was 1.0 mL min^{-1}. The thymine monomer and *cis-syn* thymine dimer peaks were monitored at 220 nm and used to calculate the resolution.

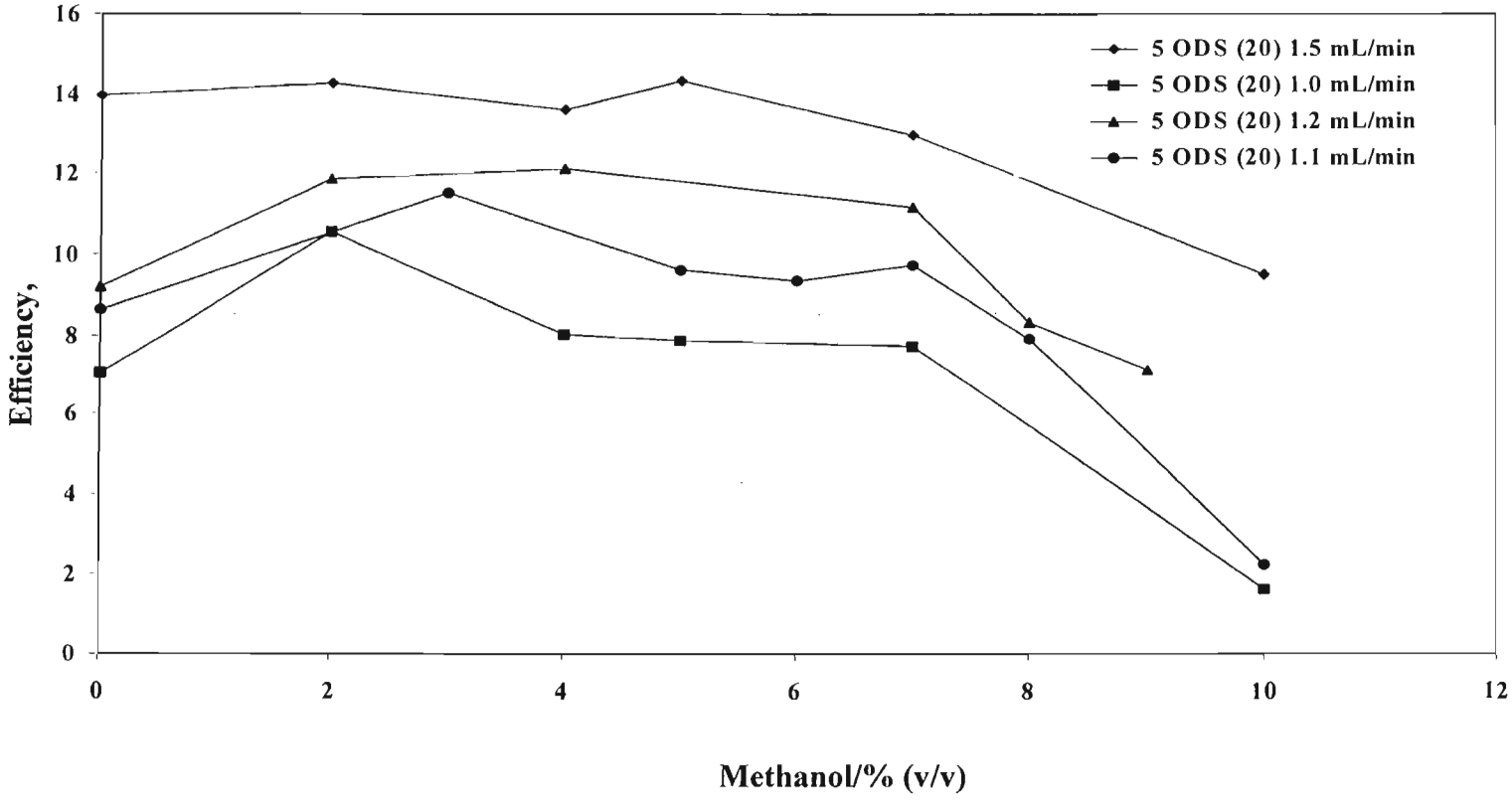

Figure 2.23: Comparison of the efficiency of the Ultracarb 5 ODS (20) column at different flow rates for varying percentages of methanol content in the mobile phase. Thymine peaks detected at 220 nm were used for the calculation.

It is apparent from the discussion above that the Ultracarb 5 ODS (20) column is superior to the other two columns. This could be due to the high carbon loading of 22% and high value of 20 for residual silanols. The Spherisorb 5 ODS (2) column has a low carbon loading (12%) and a low value of 2 for residual silanols. The carbon loading in the Nucleosil 100 C_{18} column is 14% and there are no residual silanols

The test results illustrate the superiority of one column over the other when all the conditions tested are the same besides giving an indication of the efficiency of the column. Unless the column is repacked or its dimensions changed, nothing much can be done about it regarding its general performance. Nevertheless the results serve as an indication of the suitability of the column. The ultimate goal in HPLC is resolution and thus separation of the peaks. Resolution is achieved by developing a method whose conditions are optimum. After identifying a column, two factors are important in method development, i.e. the flow rate and the composition of the mobile phase. Any method development has to take into account the time it takes to achieve separation, high column back pressures that arise from high flow rates or high methanol content in the mobile phase, and peak broadening and tailing due to too low a methanol content.

Before the Waters' Millennium software became available for calculating the different parameters of the columns, we had tried optimising the parameters based on the columns, the composition of the mobile phase and the flow rate. Initially, we chose 10% (v/v) MeOH and a flow of 1.5 mL min^{-1} on a Spherisob 5 ODS (2) column for chromatographic analysis. This was based on the recommendation by Aliwell **[74]** for the separation of thymine photoproducts on a semi-preparative column. These conditions resolved the dimers from the other photoproducts but failed to resolve the individual dimers. Figure 2.24 shows *cis-anti* dimer eluting at 9.839 minutes and thymine monomer at 18.419 minutes separated from the rest of the photoproducts. The *trans-anti* dimer at 14.939 minutes was not adequately resolved from the *trans-syn* dimer at 15.883 minutes at all as seen from the calculated resolution of 0.5491 that has been reported in Table 2.3. From the order of dimer elution given by Cadet *et al.*

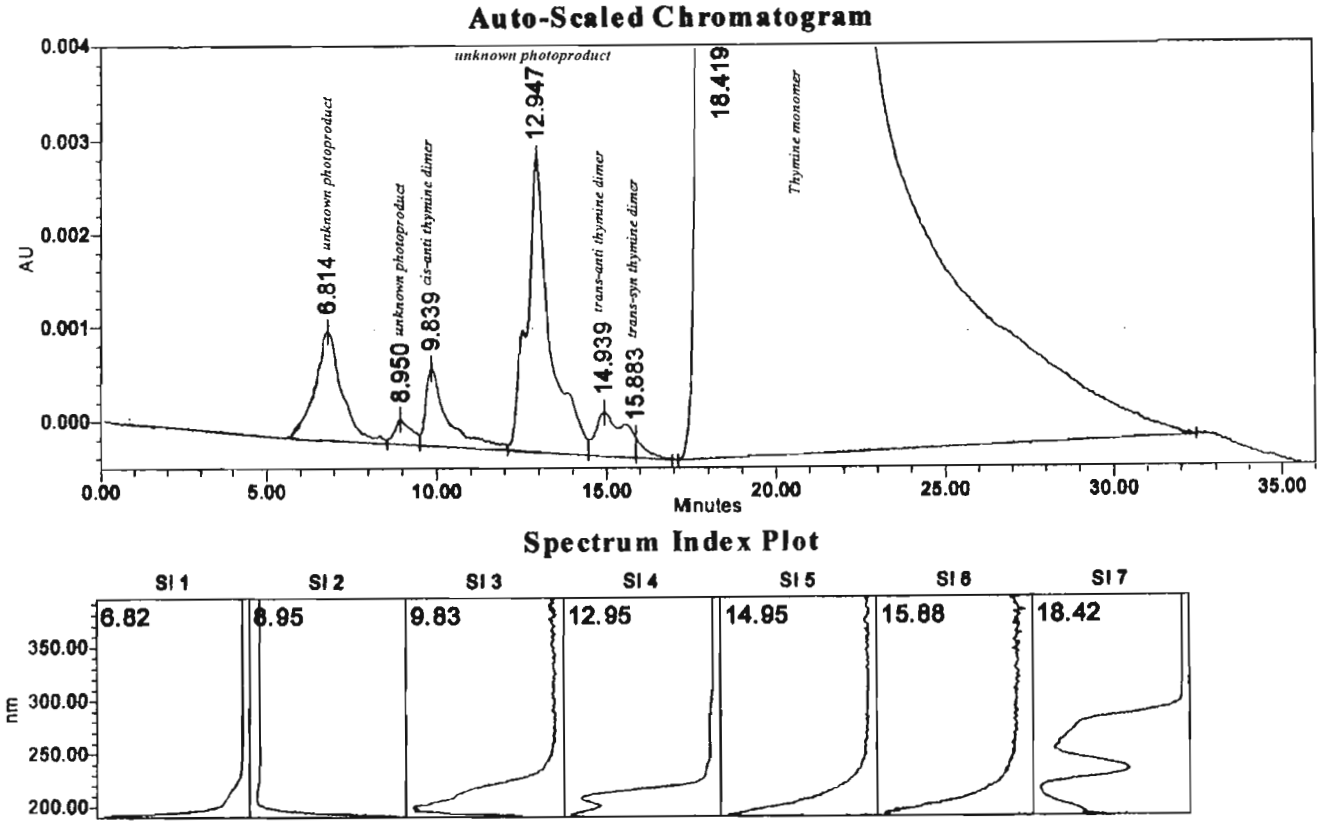

Figure 2.24: Separation of thymine/PABA photoproducts by using the Spherisorb 5 ODS (2) column, a mobile phase of 10% (v/v) MeOH with a flow rate of 1.5 mL min^{-1}. The eluted components were detected at 220 nm.

[212], *cis-syn* dimer is expected between 9.839 minutes and 14.939 minutes. The only peak that eluted between these two peaks is at 12.947 minutes. The UV spectrum of this peak indicates that it is not a thymine dimer but another photoproduct. The *cis-syn* dimer standard eluted at around the same time. This indicates that the *cis-syn* dimer was not resolved from the unknown photoproduct. In the absence of co-elution of *cis-syn* dimer and the unknown photoproduct, the former resolves from the latter with a resolution of 1.3688. Given the poor resolution of the photoproducts with the Spherisorb 5 ODS (2) column it was therefore necessary to try to optimise new separation conditions using two other columns, namely the Ultracarb 5 ODS (20) column and the Nucleosil 100 C_{18} column. The ability of each column to resolve all the four dimers and thymine with different flow rates and different percentages of methanol in the mobile phase was investigated.

Different chromatographic conditions were employed with the analytical Nucleosil 100 C_{18} column. Flow rates from 0.3 mL min^{-1} to 1.00 mL min^{-1} were used. Flow rates greater than 1.00 mL min^{-1} could not be used on this column because the maximum backpressure of the column was reached. The methanol content of the mobile phase was varied from 8% to 45% (v/v). Of all these sets of conditions, a flow rate of 0.4 mL min^{-1} and 10% (v/v) methanol was the best for this column as shown in Figure 2.25. However, these conditions gave an R value for the resolution of the thymine dimers that was far below 1.5, which is not acceptable.

The chromatographic conditions of 10% (v/v) methanol and a flow rate of 1.5 mL min^{-1} were tested for the Ultracarb 5ODS (20) column. The order of elution of the dimers was *cis-anti, cis-syn, trans-anti, trans-syn* and thymine monomer. This was verified by addition of standard solutions of *cis-syn* dimer and *trans-anti* dimer and the order given by Cadet *et al.* **[212]** who used ODS and Nucleosil columns to separate the dimers. These conditions did not yield the best resolution. It was nevertheless superior to the Nucleosil 100 C_{18} column and the Spherisorb 5 ODS (2) column for the conditions with 1.5 mL min^{-1} as the optimum flow rate. This flow rate together with a methanol content of less than 10% (v/v) gave better results but the order of elution of the dimers

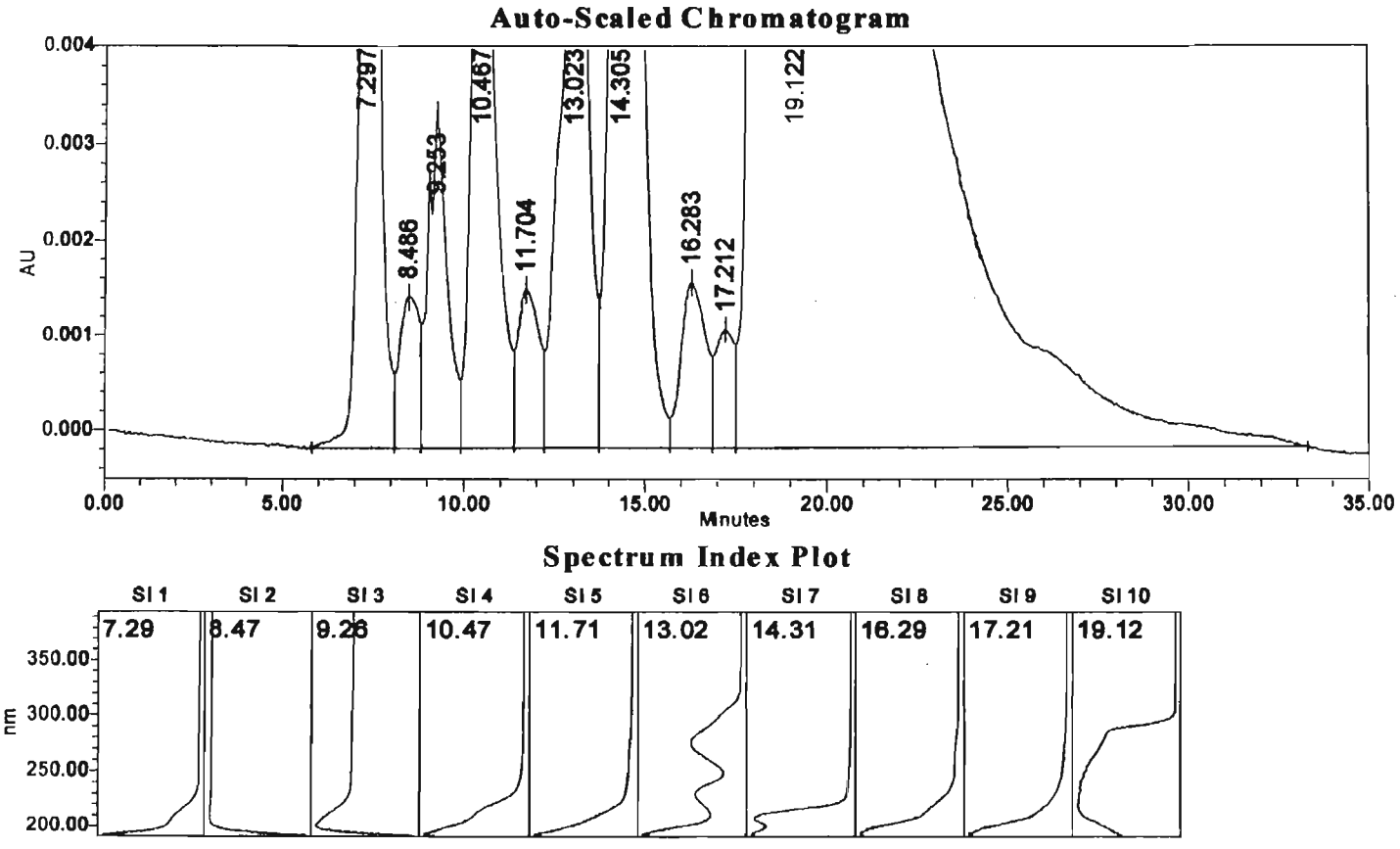

Figure 2.25: Separation of thymine/PABA photoproducts using the Nucleosil 100 C_{18} column, a 10% (v/v) MeOH mobile phase and a flow rate of 0.4 mL min^{-1} with detection at 220 nm.

changed. A methanol content of 7% (v/v) and a mobile phase flow rate of 1.5 mL min^{-1} gave the order as *cis-anti*, *trans-anti*, *trans-syn* and *cis-syn* thymine dimer and lastly thymine monomer. The order of elution in this case was slightly different from that given by Cadet *et al.* **[212]**. This is not surprising because changes in chromatographic conditions can change the order of eluting peaks **[224]**. Tables 2.3 and 2.4 show the chromatographic conditions tested for the three columns and the R values obtained for the separation. The chromatogram obtained under these conditions is shown in Figure 2.26. These conditions gave better resolution between *trans-syn* dimer and thymine monomer than what Aliwell found **[74]**. The Ultracarb 5 ODS (20) column was thus chosen for separation of the photoproducts in this work.

Table 2.3: Resolution of thymine and the photoproducts on three columns for different flow rates of the 10% (v/v) methanol-water mobile phase.

Column	R_1	R_2	R_3	R_4	R_5	Flow Rate /mL min^{-1}
Spherisorb 5 ODS (2)	3.1739	1.3688	0.5491	6.6271	Co-elution	1.5
Ultracarb 5 ODS (20)	3.1891	12.001	1.4934	1.7534	2.9949	1.5
Nucleosil 100 C18	0.9064	2.3882	0.4783	0.8196	0.5931	0.4

R_1 is the resolution between the *cis-anti* and *cis-syn* dimer peaks.

R_2 is the resolution between *trans-anti* and *cis-syn* dimer peaks.

R_3 is the resolution between the *trans-anti* and *trans-syn* dimer peaks.

R_4 is the resolution between *trans-syn* and thymine monomer peaks.

R_5 is the resolution between *trans-anti* and a photoproduct peak.

Table 2.4: Resolution of thymine and the photoproducts on the Ultracarb 5 ODS (20) column for a flow rate of 1.5 mL min^{-1} and 7% (v/v) methanol-water mobile phase.

R_1	R_2	R_3	R_4	R_5
4.2337	0.9305	9.1582	2.7595	2.1876

R_1 is the resolution between the *cis-ant* and *trans-anti* dimer peaks.

R_2 is the resolution between *trans-anti* and *trans-syn* peaks.

R_3 is the resolution between the *trans-anti* and *cis-syn* peaks.

R_4 is the resolution between *trans-anti* and a photoproduct peaks.

R_5 is the resolution between *trans-sysn* and thymine monomer peaks.

2.6.5 OPTIMUM CONDITIONS FOR SEPARATION OF PHOTOPRODUCTS

Because of differences in irradiation time and components of the samples irradiated, different photoproducts were formed in different experiments. For example, a mixture of thymine and PABA produced different photoproducts from a mixture of thymine, PABA and vitamin C. The photoproducts of the different system investigated eluted in the dimer region of the chromatogram. This necessitated alteration and re-optimisation of the chromatographic conditions as the need arose. Nevertheless, the Ultracarb 5 ODS (20) HPLC column was used for these new conditions. Tables 2.5 to 2.8 shows details of the chromatographic conditions for different components of the samples irradiated. A flow rate of 0.9 mL min^{-1} and 1% (v/v) methanol as the mobile phase were used for the separation of photoproducts from the irradiation of thymine, PABA and vitamin C and for photoproducts from the irradiation of thymine, PABA and the two vitamins for variable concentrations of vitamin C. Whenever new chromatographic conditions were adopted, injection of pure standard samples, spiking and characteristic UV spectra were used to identify dimer peaks in the chromatogram.

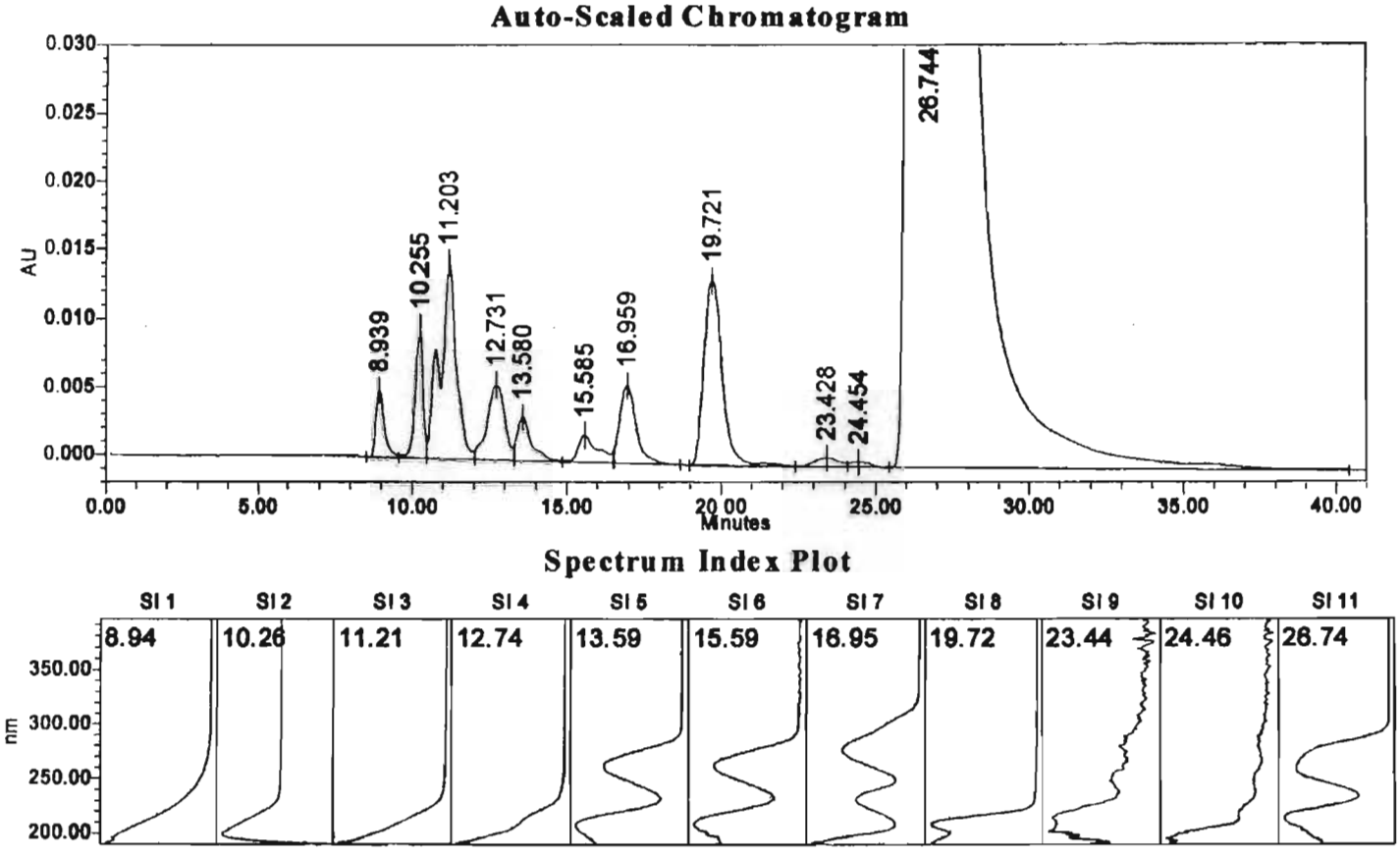

Figure 2.26: Separation of thymine/PABA photoproducts by using the Spherisorb 5 ODS (2) column, a 7% (v/v) MeOH mobile phase and a flow rate of 1.5 mL min^{-1} with detection at 220 nm.

Table 2.5: Chromatographic conditions used for the separation of photoproducts when the irradiation time was varied. The column used was an Ultracarb 5 ODS (20) semi-preparative column, the flow rate of the mobile phase was 1.5 mL min^{-1} and the eluted components were detected at 220 nm.

Components in irradiated sample	Methanol content in the mobile phase/% (v/v)
Thymine + PABA	10
Thymine + PABA	7
Thymine + PABA	5
Thymine + PABA + vitamin E	4
Thymine + PABA + vitamin C	4
Thymine + PABA + vitamins C + E	4

Table 2.6: Chromatographic conditions used for the separation of photoproducts when the concentration of PABA was varied and the irradiation time was 6 hours. The column used was an Ultracarb 5 ODS (20) semi-preparative column, the flow rate of the mobile phase was 1.5 mL min^{-1} and the eluted components were detected at 220 nm.

Components in irradiated sample	Methanol content in the mobile phase/% (v/v)
Thymine + PABA	4
Thymine + PABA+ vitamin E	4
Thymine + PABA+ vitamin C	4
Thymine + PABA + vitamins C + E	1

Table 2.7: Chromatographic conditions used for the separation of photoproducts when the concentration of thymine was varied and the irradiation time was 6 hours. The column used was an Ultracarb 5 ODS (20) semi-preparative column, the flow rate of the mobile phase was 1.5 mL min^{-1} and the eluted components were detected at 220 nm.

Components in irradiated sample	Methanol content in the mobile phase/% (v/v)
Thymine + PABA	4
Thymine + PABA+ vitamin E	4
Thymine + PABA+ vitamin C	4
Thymine + PABA + vitamins C + E	4

Table 2.8: Chromatographic conditions used for the separation of photoproducts when the concentration of vitamin E was varied and time was 6 hours. The column used was an Ultracarb 5 ODS (20) semi-preparative column, the flow rate of the mobile phase was 1.5 mL min^{-1} and the eluted components were detected at 220 nm.

Components in irradiated sample	Flow rate/ mL min^{-1}	Methanol content in the mobile phase/% (v/v)
Thymine + PABA+ vitamin E	1.5	4
Thymine + PABA+ vitamin C + E	1.5	1

2.6.6 COLUMN RECONDITIONING AND CLEANING

For optimum column performance, in particular to obtain consistency in the retention times of peaks, the Ultracarb 5 ODS (20) column had to be

reconditioned periodically. The gradient mode of the HPLC was used for column reconditioning and cleaning. The following gradient was used for reconditioning the column: water for 30 minutes at 1.5 mL min^{-1}, water to methanol (MeOH) over 5 minutes at 1.5 mL min^{-1}, MeOH for 30 minutes at 1.5 mL min^{-1}, MeOH to acetronitrile (ACN) over 5 minutes at 1.5 mL min^{-1}, ACN for 30 minutes at 1.5 mL min^{-1}, ACN to tetrahydrofuran (THF) over 5 minutes at 1.5 mL min^{-1}, and THF for 30 minutes at 1.5 mL min^{-1}. The gradient procedure was then reversed. During this gradient, the helium sparge rate was set at 100 mL min^{-1}. Whenever a blank injection indicated the presence of PABA, the column was washed with 100% methanol at a flow rate of 1.5 mL min^{-1} until no PABA appeared in the chromatogram of a blank injection.

2.6.7 DETERMINATION OF ELUTION ORDER FOR THE THYMINE DIMERS AND THYMINE MONOMER

Different irradiation times and solution compositions were used in the course of the experiments described herein. Because of this, different photoproducts besides thymine dimers were formed in different systems. This necessitated changes in the chromatographic conditions so that separation could be achieved. Retention times of standards of the thymine monomer and the two thymine dimers synthesised were used to locate unreacted monomer and the dimer peaks respectively whenever the chromatographic conditions were changed. The position of the thymine monomer peak was easy to locate because of its UV-spectrum and the height of its peak. It was always the tallest peak. The four dimers have similar UV-absorption spectra. Hence their UV spectra were not sufficient to identify their positions. We first had to identify the *cis-syn* and *trans-anti* thymine dimers by eluting their pure standards at the relevant conditions. By elimination we then assumed that the *cis-anti* dimer elutes before the *trans-syn* dimer. This was based on the order given by Cadet *et al.* **[213]**. At times spiking the sample with the standards was also used to confirm the retention times. Figures 2.27 to 2.30 show chromatograms and UV spectra for standards of *cis-syn* and *trans-anti* dimers and thymine for some of the conditions used in the separation of the photoproducts. Table 2.9 shows the eluting order for the chromatographic conditions used.

Table 2.9: Elution order of thymine dimers and thymine monomer together with the chromatographic conditions used on the Ultracarb 5 ODS (20) semi-preparative column. The eluted components were detected at 220 nm.

Flow rate/ mL min^{-1}	Methanol content in the mobile phase/% (v/v)	Elution order
1.5	10	*cis-anti, cis-syn, trans-anti, trans-syn* and thymine
1.5	7	*cis-anti, cis-syn, trans-anti, trans-syn* and thymine
1.5	5	*cis-anti, cis-syn, trans-anti, trans-syn* and thymine
1.5	4	*cis-anti, cis-syn, trans-anti, trans-syn* and thymine
1.5	1	*cis-anti, trans-syn, cis-syn,* thymine and *trans-anti*
0.9	1	*cis-anti, trans-syn, cis-syn,* thymine and *trans-anti*

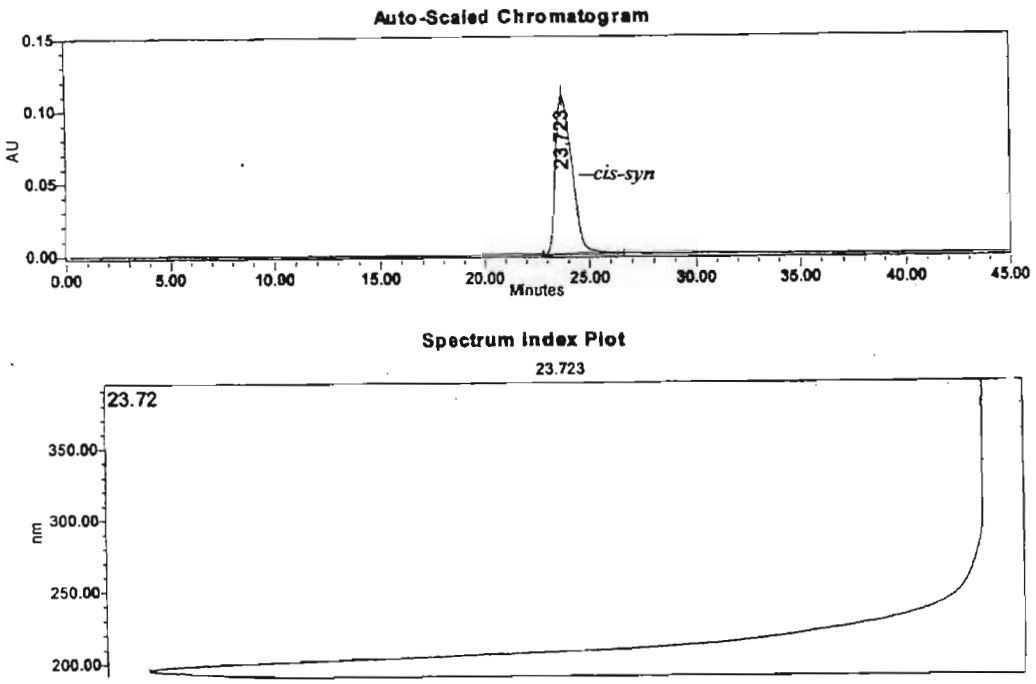

Figure 2.27: Chromatogram and UV-absorption spectrum for *cis-syn* dimer standard eluted with mobile phase of 5% (v/v) methanol at a flow rate of 1.5 mL min^{-1} on an Ultracarb 5 ODS (20) column with detection at 220 nm.

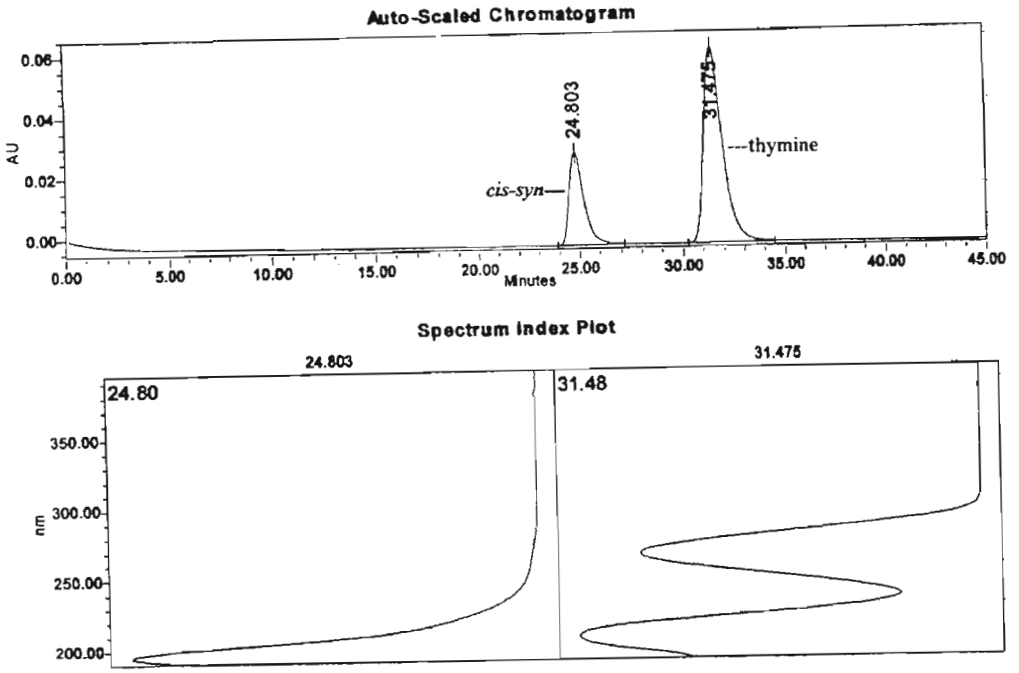

Figure 2.28: Chromatogram and UV-absorption spectra for *cis-syn* dimer and thymine standards eluted with a 4% (v/v) methanol mobile phase at a flow rate of 1.5 mL min^{-1} on an Ultracarb 5 ODS (20) column and detected at 220 nm.

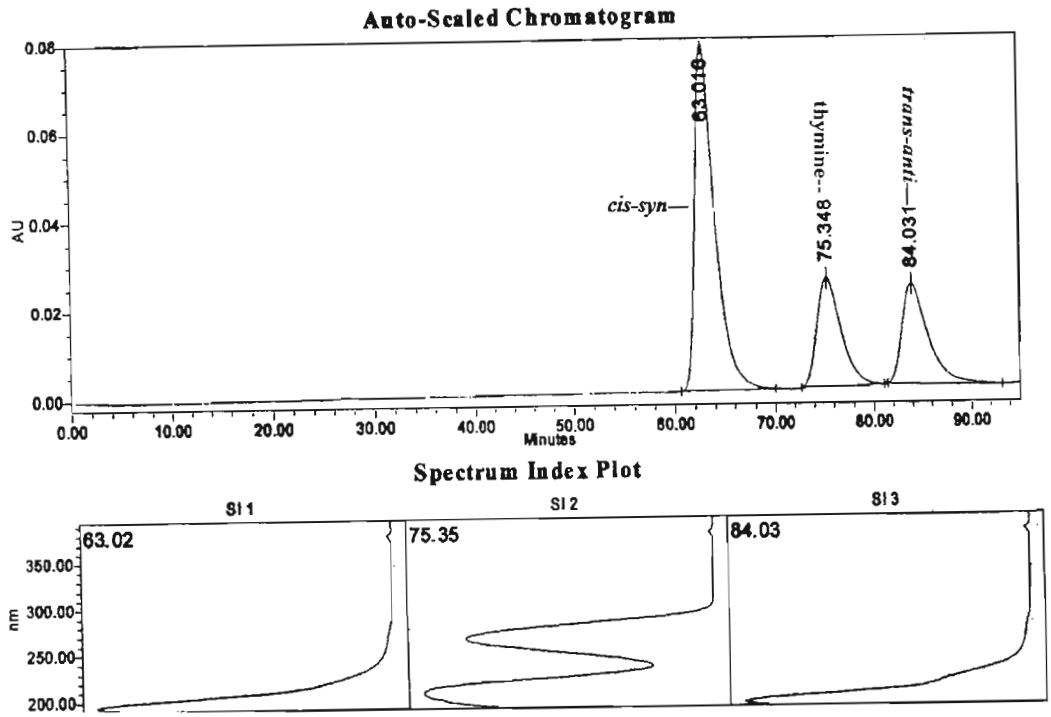

Figure 2.29: Chromatogram and UV-absorption spectra for *cis-syn* dimer, thymine and *trans-anti* dimer standards eluted with a 1% (v/v) methanol mobile phase at a flow rate of 0.9 mL min^{-1} on an Ultracarb 5 ODS (20) column and detected at 220 nm.

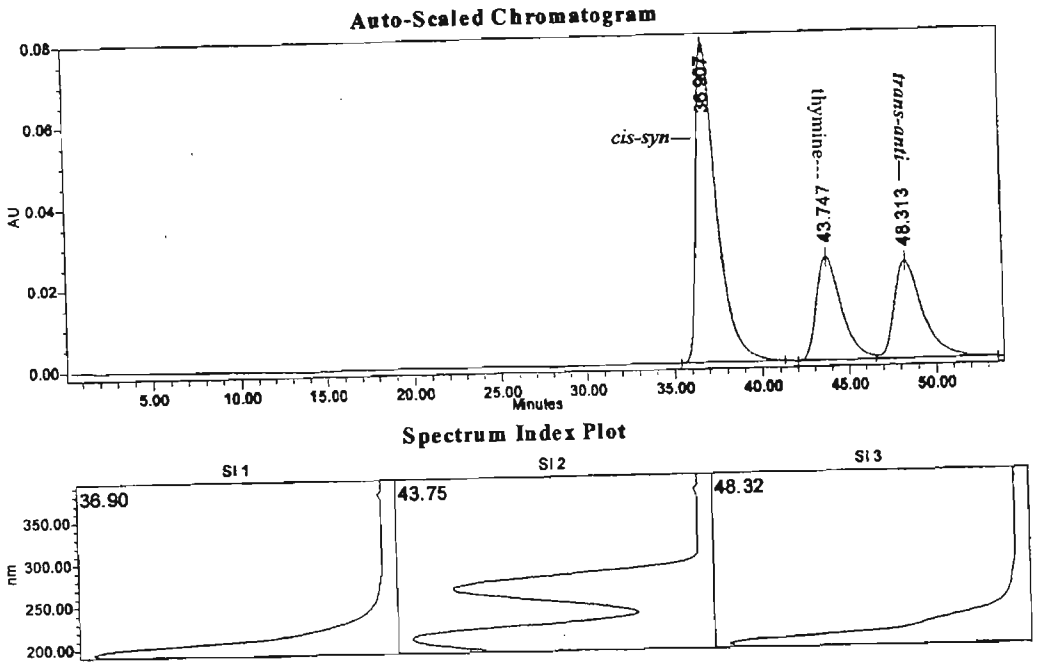

Figure 2.30: Chromatogram and UV-absorption spectra for *cis-syn* dimer, thymine and *trans-anti* dimer standards eluted with a 1% (v/v) methanol mobile phase at a flow rate of 1.5 mL min^{-1} on an Ultracarb 5 ODS (20) column and detected at 220 nm.

2.7 QUANTIFICATION OF THYMINE DIMERS

A number of methods can be employed to quantify the thymine dimers formed. Each of them was has limitations that are discussed below.

One method involves determining the amount of dimer that photochemically converts back to the monomer by measuring the absorbance at 265 nm, the maximum absorbance of the monomer **[35, 36]**. The assumptions here are that the conversion goes to completion and that the conversion to monomer is the only reaction that takes place. In practice this does not occur and hence the method is prone to inaccuracy.

Isotope labelling is an alternative method. Thymine monomer is labelled with a radioactive isotope and the amount converted to the dimer followed by a

counter **[225]**. The cost of this method is prohibitive and suitable laboratory infrastructure for working with radioisotopes is required.

Quantitation of thymine dimer using HPLC has been previously reported by Cadet *et al.* **[212]**. They used a reversed-phase ODS C_{18} column with a 1% THF and 99% water mixture to separate the photoproducts. Aliwell **[74]** used an Ultracarb 5 ODS (2) column and 10% (v/v) methanol acidified with perchloric acid to delay elution of PABA **[74]**. Paterson-Jones used 15% (v/v) methanol **[76]**. Kilfoil achieved optimum separation with a Spherisorb ODS 1 column and water containing organic modifiers (MeOH, ACN or THF) in the range of 0% to 5% (v/v) as mobile phases **[226]**. In all these three cases there was a problem of co-elution of the *trans-syn* dimer with the monomer.

Individual concentrations of the dimers can be calculated accurately only if the peaks in the chromatogram are completely resolved with $R \geq 1.5$ and standards are available for calibration. The method of standard addition also requires standards of all the dimers. The only standards that can be prepared easily are those of *cis-syn* and *trans-anti* dimers. In this experimental work, the dimer concentrations in the irradiated samples were determined by using peak area obtained from HPLC analyses. For this purpose a calibration graph is necessary. The concentration of a component is directly proportional to the area or the height of the peak of the component separated on the HPLC, which in turn is directly proportional to the absorbance, i.e.

$$peak\ area = K \times concentration \qquad 2.8$$

where K is a proportionality constant that can be determined from the gradient of a graph of peak area versus dimer concentration. This relationship in the case of dimer concentration, gives concentrations of individual dimers and not the total dimer concentration. Therefore, standards would be required for each dimer isomer. Because of experimental difficulties in the preparation of other dimers **[77]** and the fact that the *trans-anti* thymine dimer is less stable thermally and in solution than *cis-syn* thymine dimer **[227]**, only the *cis-syn* thymine dimer standard could be used for calibration and the total dimer

concentration determined by correction of the dimer peak areas using the appropriate molar absorption coefficient. The formulas and parameters used for the correction of the individual dimer concentrations are explained in Section 2.7.1.

The *cis-syn* thymine dimer prepared by the ice irradiation method was used to prepare an assumed concentration of 1 x 10^{-3} M since some of the monomer co-precipitated with the dimer. Absorbance measurements were made at 235 nm and 265 nm because the molar absorption coefficients for the *cis-syn* dimer and thymine are known at these wavelengths **[35, 75]**.

For the solution containing thymine and *cis-syn* thymine dimer the following simultaneous equations that obey the Beer-Lambert law were solved to obtain concentrations of the dimer and the monomer:

$$A_{235} = [\varepsilon_{1,235} \times C_1 \times I_1] + [\varepsilon_{2,235} \times C_2 \times I_2] \qquad 2.9$$

$$A_{265} = [\varepsilon_{1,265} \times C_1 \times I_1] + [\varepsilon_{2,265} \times C_2 \times I_2] \qquad 2.10$$

where A and I represent absorbance and the pathlength of the cell, respectively. The value of I is always the same since the components are contained in the same cuvette. Component 1 (*cis-syn* thymine dimer) and component 2 (thymine) are represented by 1 and 2, respectively. C_1 and C_2 are their respective concentrations. The values of molar absorption coefficients in the above equations are as follows:

$\varepsilon_{1,235}$ = 1.5 x 10^3 M^{-1} cm^{-1}, $\varepsilon_{2,235}$ = 2.24 x 10^3 M^{-1} cm^{-1}, $\varepsilon_{1,265}$ = 87 M^{-1} cm^{-1} and $\varepsilon_{2,235}$ = 7.89 x 10^3 M^{-1} cm^{-1} **[35, 75]**.

The concentration of the *cis-syn* dimer was found to be 9.254 x 10^{-4} M. Dimer solutions of concentrations ranging from 9.254 x 10^{-4} M to 4.336 x 10^{-5} M were prepared by serial dilution. Several calibration curves for the *cis-syn* dimer concentrations for different chromatographic conditions were obtained using

this range of concentrations. Table 2.10 shows the chromatographic conditions used and the gradients obtained from the calibration curves.

Table 2.10: Chromatographic conditions used for obtaining the thymine dimer calibration graph together with the gradients obtained from the graphs.

Flow Rate/mL min^{-1}	MeOH/% (v/v)	Gradient/M^{-1}
1.5	10	21718
1.5	5	11723
1.5	4	15301
1.5	1	13103
0.9	1	22045

The calibration curves in the above table gave a detection limit of 4.047×10^{-7} M for the PDA used in our experiment. Figure 2.31 shows an example of a calibration curve obtained using one of the sets of conditions given in Table 2.10.

2.7.1 CALCULATION OF TOTAL DIMER CONCENTRATION FROM THE INDIVIDUAL DIMER PEAK AREAS

Separation and quantification of photoproducts was performed using HPLC with UV detection at 220 nm. At this wavelength, the dimers and other photoproducts are easily detected. The procedure used to obtain the total dimer concentration is detailed in this section.

The molar absorption coefficients of the dimers at 220 nm are shown in Table 2.11. The percentage distributions of the four dimers were first calculated based on their peak area.

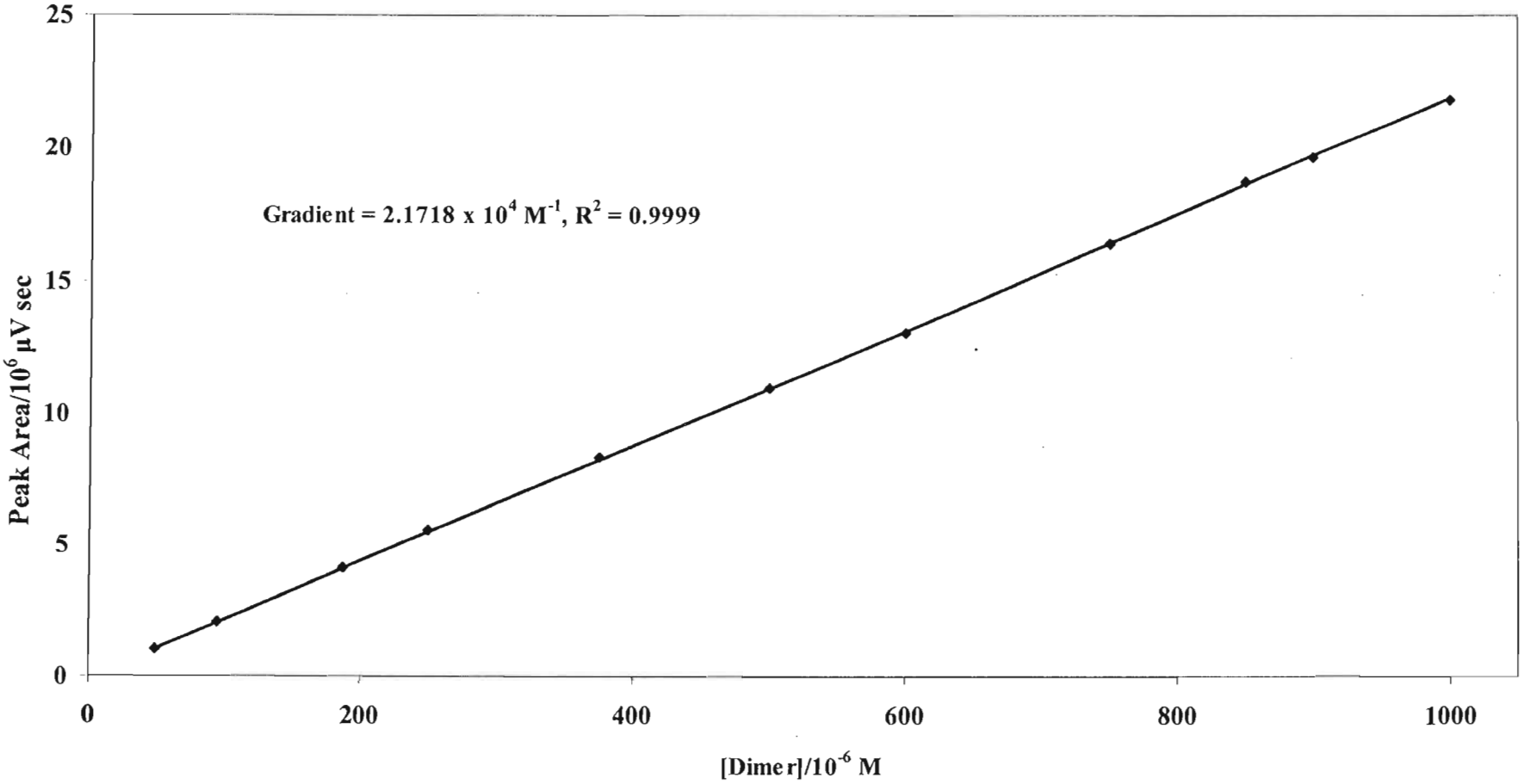

Figure 2.31: Calibration curve for aqueous solutions of *cis-syn* thymine dimer eluted on an Ultracarb 5 ODS (20) column with a 10% (v/v) MeOH mobile phase at a flow rate of 1.5 mL min^{-1}. Detection of the eluted components was at 220 nm. The points represent single injections of the standards.

Table 2.11: Molar absorption coefficient of thymine dimers at 220 nm **[35]**.

Dimer	ε at 220 nm/ $M^{-1}cm^{-1}$
cis-anti	5.5×10^3
cis-syn	4.6×10^3
trans-anti	6.7×10^3
trans-syn	5.4×10^3

From the percentage distribution, a weighted average molar absorption coefficient for total dimer was calculated according to Equation 2.11.

$$\varepsilon_{dimer} = \left(\frac{\%_{c-a} \times \varepsilon_{c-a}}{100}\right) + \left(\frac{\%_{c-s} \times \varepsilon_{c-s}}{100}\right) + \left(\frac{\%_{t-a} \times \varepsilon_{t-a}}{100}\right) + \left(\frac{\%_{t-s} \times \varepsilon_{t-s}}{100}\right) \qquad 2.11$$

where c-a, c-s, t-a and t-s respectively stand for *cis-anti, cis-syn, trans-anti* and *trans-syn* thymine dimers.

By using Equation 2.12, the proportionality constant k_{dimer}, needed to relate the total dimer peak area to the total dimer concentration in Equation 2.13 could be calculated thus

$$\frac{k_{dimer}}{k_{c-s}} = \frac{\varepsilon_{dimer}}{\varepsilon_{c-s}} \qquad 2.12$$

$$\text{Total dimer concentration} = \frac{\text{Total dimer peak area}}{k_{dimer}} \qquad 2.13$$

This is based on the fact that peak area is proportional to absorbance, which in turn is proportional to the sum of molar absorption coefficient and concentration. For all the photodimerisation experiments performed, dimer yield refers to total dimer yield for all four dimer stereoisomers calculated as described above.

2.8 PABA-PHOTOSENSITISED THYMINE DIMERISATION EXPERIMENTS

In the photosensitised dimerisation reaction, the main process is the absorption of UV radiation by PABA at wavelength not absorbed by the free base or any of the other constituents. In order to achieve this, the solutions were irradiated as described in Section 2.3 with the Osram HBO 500 W high pressure mercury lamp and Pyrex filter combination ($\lambda > 300$ nm). Figure 2.32 shows UV spectra of aqueous solutions of PABA, thymine and vitamins C and E at pH 3.0. This shows that only PABA and vitamin E absorb light at wavelengths greater than 300 nm.

2.8.1 CHOICE OF EXPERIMENTAL CONDITIONS

In our experiments, α-tocopherol acetate and ascorbic acid were used. Therefore whenever reference is made to vitamins E and C, it implies α-tocopherol acetate and ascorbic acid respectively. A number of variables were examined in the PABA-photosensitised dimerisation of free thymine base. These were the initial PABA, thymine, and antioxidant concentrations and irradiation time.

Thymine dimer yields were determined at pH 3.0 for the following reasons. PABA's UV absorption and other physical properties are strongly dependent on pH. At pH 6.88 the absorbance of PABA is 0.094 whereas at pH 3.97 the absorbance is 1.183 **[73]**. It absorbs UV radiation intensely at pH 3 **[65, 73]**. As explained in Chapter 1, in aqueous solution PABA exists as a neutral molecule, in ionised or unionised form **[72]**. Given its pK_a value, PABA exists in an ionised form at pH 7 and is unionised at pH 3 **[228]**. Some literature reports indicate that it exists as a zwitterion at low pH values **[73]**. At pH values closer to the pK_a value both ionised and unionised forms exist. The form in which PABA exists affects its self-quenching efficiency although the process of

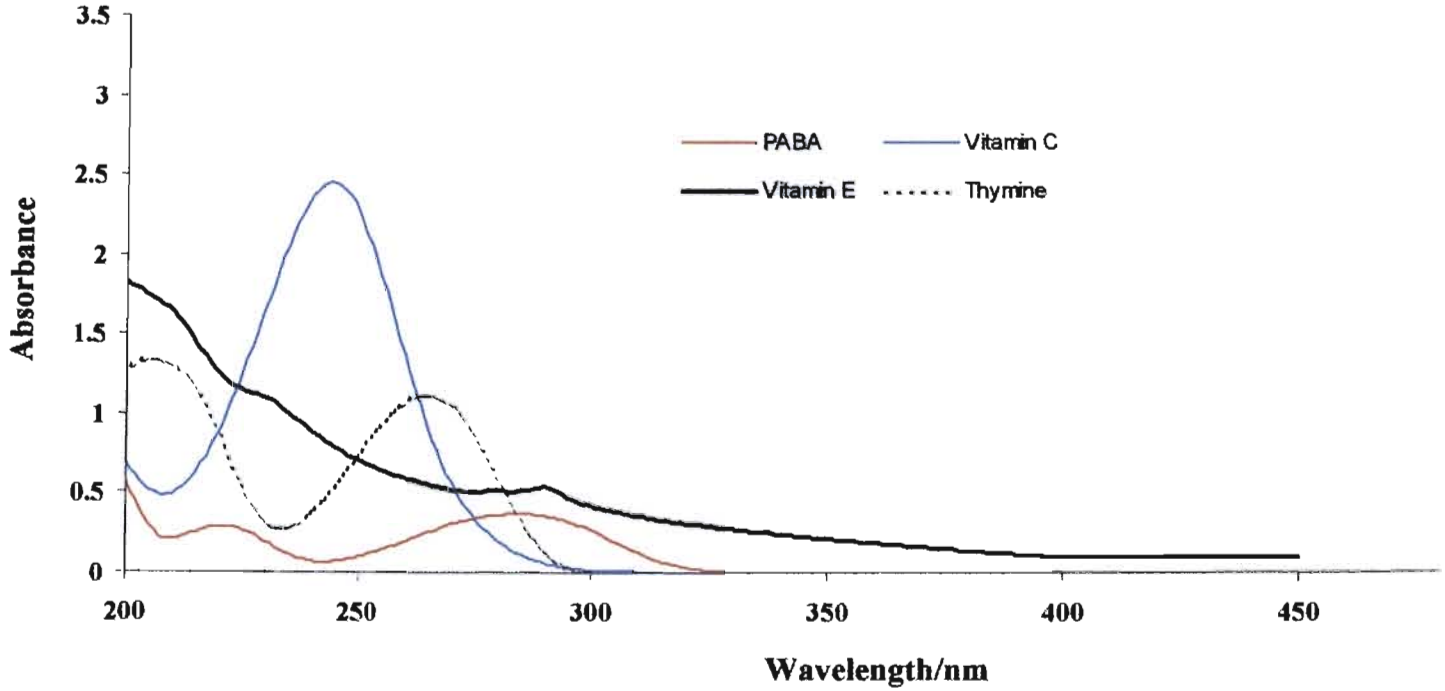

Figure 2.32: UV spectra of aqueous solutions of PABA, thymine, and vitamins C and E at pH 3.0 obtained in a 10 mm pathlength quartz cell.

photosensitisation in the two forms is essentially the same **[74]**. In unionised form, self-quenching between triplet PABA and ground state PABA is very efficient, $k = 5.0 \times 10^9 s^{-1}$ **[65]**. Nevertheless, at concentrations below 3×10^{-4} M, the unionised form is an efficient photosensitiser **[65]**. Because of electrostatic repulsion in the ionised form, self-quenching is very inefficient **[65, 74]**.

PABA's photosensitising efficiency of free thymine base in aqueous solution was found to depend on pH and concentration of PABA **[65]**. While using radiation of wavelength greater than 300 nm, Rutherford *et al.* **[65]** found that at low PABA concentrations, there is competition for light between PABA and thymine.

In solution thymine can deprotonate to form a mono-anion, with a pK value of 9.9. Thus low concentrations of thymine ion are present at at low pH **[75]**. Paterson-Jones **[76]** and Malone **[77]** found in their experiments while studying acetone-photosensitised thymine dimer formation that the deprotonation of thymine has an effect on the dimer yield and that dimer yield decreased with an increase in pH. This implies that at an acidic pH of 3, there should be a relatively higher thymine dimer yield **[76]**.

Cis-syn thymine dimer has pK_1 and pK_2 as 10.65 and 12.45 respectively **[35]**. Hence, the rate at which it reverts to monomer depends on pH. Once formed in solution, it can revert to the monomer depending on the pH. At high pH, the cyclobutyl ring of the dimer opens up whereas at low pH it closes. This implies that it is most stable in solutions of low pH **[78]**.

The diffusion-controlled mechanism that involves triplet energy transfer accounts for only a fraction of the total dimer yield and applies only to low thymine concentrations, of the order of 10^{-4} M to 10^{-3} M. At concentrations higher than 1×10^{-3} M, the mechanism does not apply but instead base stacking accounts for the high dimer yield **[34]**. Base stacking is more pronounced in aqueous solution **[34]**. At thymine concentrations below 1×10^{-3} M, base stacking is known to be negligible **[34]**. Nevertheless, both Rutherford **[65]** and

Aliwell **[74]** showed that when using a thymine solution having a concentration of 1 x 10^{-2} M in the presence of PABA, the reaction mechanism can proceed via a triplet state. In our studies, a thymine concentration between 1 x 10^{-3} M and 1 x 10^{-2} M was used. Therefore, at these thymine concentrations, the dimerisation should be via the triplet energy transfer route with PABA as the photosensitiser.

The absorbance of ascorbic acid changes with variation in pH **[229]**. As mentioned earlier in Chapter 1, ascorbic acid is most stable at a pH range of 4 to 6. This requires that the pH of the mobile phase be acidic. Nevertheless, aqueous solutions of ascorbic acid are very prone to oxidation by air and the intensity of its UV absorption band can drop by even a half in a few hours because of oxidation. However, the absorption band is persistent at acidic pH **[161, 229]**. The extent of oxidation is dependant on time and temperature. Therefore, precautions should always be taken to exclude a rise in temperature **[161, 230]**. From Figure 2.32, vitamin C does not absorb above 300 nm. Therefore, it is appropriate for our studies in which only PABA should absorb radiation.

From the preceding paragraphs, it can be concluded that a pH value of 3 is optimal for our investigation. This pH ensures that PABA-photosensitised dimerisation of thymine is via the triplet state, that UV absorption by PABA is maximum, and that there is minimum deprotonation of thymine and hence more dimer yield. It also ensures that the dimer formed does not convert back to the monomer and lastly it ensures stability of ascorbic acid throughout the experiment.

Free vitamin E is available only as an oil that is insoluble in aqueous solvents. It is affected by the oxidizing influence of air and light but its acetate salt is not **[231]**. The acetate is the water-soluble formulation of vitamin E available on the market. The international standard unit of vitamin E is equal to one milligram of dl-α-tocopheryl acetate **[232]**. The maximum absorption of free vitamin is at 290 nm whereas for the acetate it is at 284 nm **[152]**. The latter is commonly used in sunscreen formulations. The acetate is thus considered

representative of vitamin E activity. α-Tocopherol acetate was hence chosen in our investigation to determine the role of vitamin E in thymine dimer formation.

Concentrations for working solutions of each of the components in the samples were arrived at after considering the validity of the Beer-Lambert Law, the reaction mechanism of photosensitisation, the detection limit of the instruments used for photoproduct analysis and the stability of ascorbic acid. At very high reagent concentration, when the absorbance of the sample is more than 1.5, deviations from the Beer-Lambert Law occur. UV spectra for individual solutions of vitamin E and vitamin C were recorded to determine the maximum working concentration that obeyed the law. A choice had to be made between a low concentration that obeyed the Beer-Lambert law and a high concentration that ensured a sufficient amount to participate in the dimerisation reaction. Section 2.8.2 details the experimental procedure used to arrive at the various concentrations of reagents used in the investigation. Suitable irradiation times were chosen to investigate the trend in dimer formation with change in photon flux.

A preliminary investigation on the stability of ascorbic acid solutions was also carried out. For this purpose, the pH was maintained at 3, the solutions were purged with nitrogen and protected from light by aluminium foil. Whenever the solution was not used it was stored in the refrigerator to prevent a rise in temperature. Analyses were carried out by means of HPLC at detection wavelengths of 220 nm, 265nm and 245 nm. We chose 220 nm because this is the wavelength at which we detected thymine photoproducts whereas the other two wavelengths are where ascorbic acid absorbs. Since peak area is proportional to the amount of vitamin C present, any decrease in peak area would give an indication of the extent of its instability. Figure 2.33 shows the peak area of ascorbic acid as a function of time from when the solution was prepared. It is evident that at pH 3, it remains stable for over 67 hours if the above-mentioned precautions are taken. This length of time is more than what is required for the preparation of the photochemical experiments, and analysis.

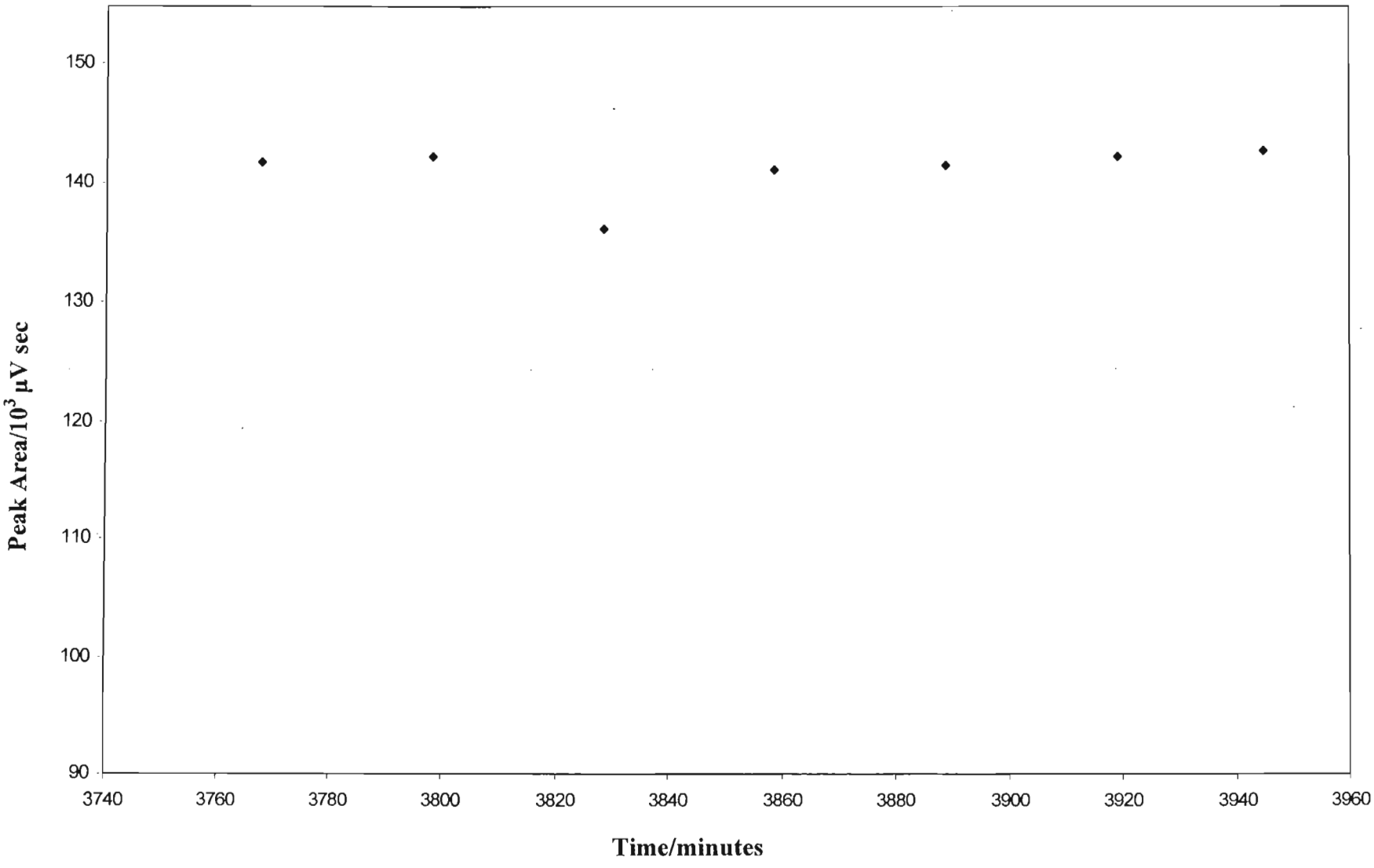

Figure 2.33: Stability of an aqueous solution of vitamin C at pH 3.0 over time. The peaks were detected at 220 nm by HPLC.

2.8.2 EXPERIMENTAL PROCEDURE

To adjust the pH of the solutions, Aliwell [74] used a phosphate buffer but abandoned it because photoproducts formed with the buffer itself. For this reason we did not use any buffer. All solutions for irradiation were adjusted to pH 3.0 by addition of hydrochloric acid. Prior to irradiation all samples were purged with nitrogen for fifteen minutes. The samples were then irradiated at wavelengths greater than 300 nm by using the Osram HBO 500 W high-pressure mercury lamp and Pyrex filter combination as described in Section 2.3. Irradiated samples were always stored in a refrigerator if they could not be analysed immediately. All irradiated solutions that contained ascorbic acid were analysed immediately to prevent the formation of decomposition products from ascorbic acid itself and avoid introducing new products that did not form as a result of the desired photolysis reactions.

The wavelength of maximum absorption for vitamin E acetate is 284 nm, for ascorbic acid they are 245 nm and 265 nm and for PABA 284 nm at pH 3.0 Photoproducts in all the experiments performed were investigated at 220 nm by means of HPLC and a PDA detector. Photoproducts could not be detected at other wavelengths. The column used was the reverse phase Ultracarb 5 ODS (20) with different percentages of methanol in the mobile phase as indicated in Section 2.6.5. These different compositions of the mobile phase were acidified, maintained at pH 3.0 by using perchloric acid and used in all the HPLC analyses carried out. Characteristic retention times and UV spectra of standards were the basis for dimer identification. Dimer yields were calculated as described in Section 2.7. The *cis-syn* thymine dimer calibration curves were obtained by single injections and used for quantitation of the total dimer yield. Typically three injections were done and by use of Q-test and standard deviation outliers were rejected. Details of the standard deviations are in Appendix C.

2.8.3 CONTROL EXPERIMENTS

To compare the photochemistry of the substrates involved in our investigation, sets of control experiments were prepared in the same way as the samples. The concentrations used were the same as those of the samples. Long irradiation times were used in order to determine the maximum effect and so as to easily detect any photoproducts. Solutions were adjusted to a pH of 3.0. They were then purged with nitrogen for fifteen minutes to remove dissolved oxygen. They were protected from light by covering the flasks with aluminium foil. They were irradiated and analyzed by HPLC with the reverse-phase Ultracarb 5 ODS (20) column. Standards of *cis-syn* and *trans-anti* thymine dimers, thymine, PABA, vitamin E and vitamin C were used to locate their peaks. Different chromatographic conditions were used for the separation of the photoproducts formed in the controls or for identifying the peaks of un-irradiated controls. The conditions used are those indicated in Section 2.6.5 and in Tables 2.5 to 2.8. The controls irradiated are as shown in Table 2.12.

Table 2.12: Control experiments that were irradiated for 24 hours with Osram HBO 500 W high pressure mercury lamp combined with a 10 mm thick Pyrex filter and analysed by HPLC with PDA detection at 220 nm.

1-Thymine	5-Thymine and vitamin C
2-PABA	6-Thymine and vitamin E
3-Vitamin E	8-PABA and vitamin C
4-Vitamin C	9-PABA and vitamin E

10-Vitamin E and vitamin C
11-Vitamin C and Vitamin E and Thymine
12-Vitamin C and Vitamin E and PABA

Figure 2.34 shows the chromatogram of the photoproducts formed when thymine is irradiated alone. It is evident that even in the absence of PABA, thymine can give some dimers and other photoproducts. The absorbance of these dimers and photoproducts is very small and therefore indicative of the

yields of dimer and other photoproducts. While irradiating thymine at 265 nm in the absence of PABA, Aliwell *et al.* **[233]** showed that the yield of unknown photoproducts was below the detectable limit. In other studies, Aliwell *et al.* **[234]** showed that the rate of dimer formation decreases and approaches zero for longer irradiation times. They also suggested the possibility of photoproducts that absorb UV light but are unable to photosensitise dimer formation or do so with very low efficiency.

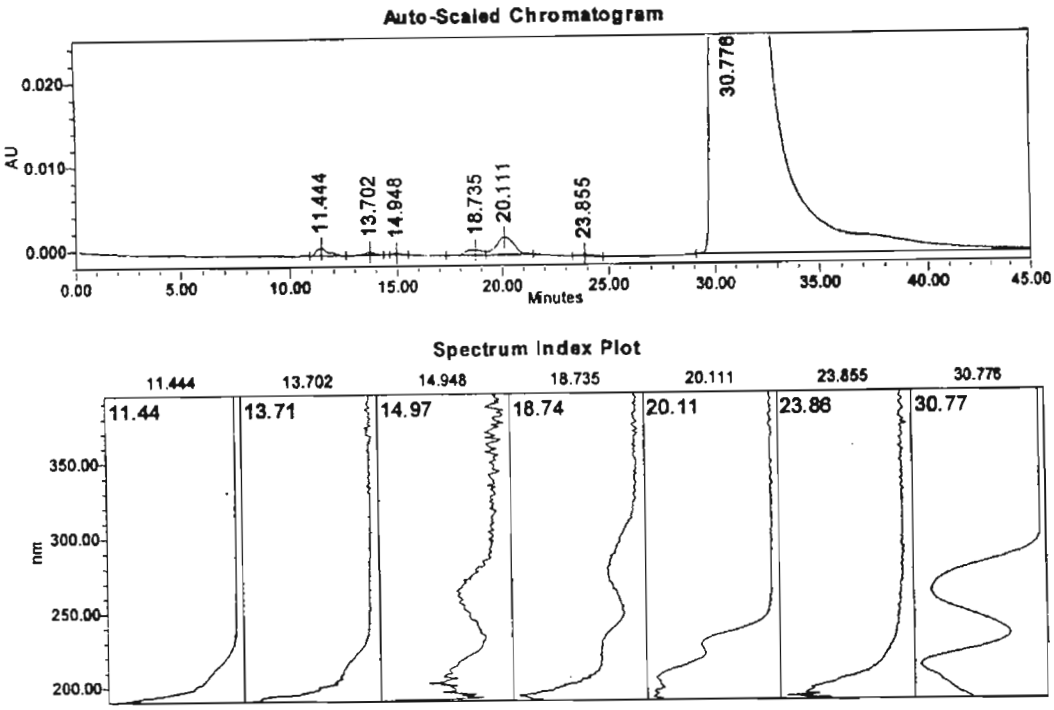

Figure 2.34: HPLC chromatogram of [thymine] = 1 x 10^{-2} M solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on an Ultracarb 5 ODS column with a mobile phase of a 5% (v/v) methanol at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

The peaks at 23.86 and 11.44 minutes are for *cis-syn* and *trans-syn* dimers respectively. Peaks for the other two dimers are not present in the chromatogram. Either they were not formed at all or they were formed in amounts below the detection limit of the PDA (4.047 x 10^{-7} M). Peaks at 13.71, 14.97, 18.74 and 20.11 minutes are for unknown photoproducts resulting from the irradiation of thymine. The peaks at 13.71 and 20.11 minutes are similar to those of thymine dimer thereby strongly suggesting that they could be for compounds resulting from the reaction of the 5,6-double bond of thymine. The peak at 14.97 minutes shows a UV spectrum with two absorptions at around

210 nm and 265 whereas the peak at 18.74 minutes shows absorption at around 225 nm and 265 nm. The UV spectra for these two peaks resemble that of thymine peak at 30.77 minutes. This implies that the products represented by the two peaks could have lost the conjugation present in thymine due to the 5,6-double bond and the C(4) carbonyl group **[233]**. Some of the possible non-dimer products are 5,6-dihydrothymine, 5-dihydroxy-5,6-diyhdroxythymine and 5-hydroxymethyluracil **[233]**. Since UV spectra provide very little evidence of the identity of the peaks, further analysis techniques such as HPLC-mass spectrometry is required to give more conclusive information. It is evident that direct irradiation of thymine with light of wavelengths greater than 300 nm can produce dimers besides other photoproducts even in the absence of a photosensitiser like PABA. Nevertheless the yield is very small. The presence or absence of photoproducts in this chromatogram and the chromatogram in Figure 2.35 enabled identification of the source of a given photoproduct.

Irradiation of PABA alone produced no detectable photoproducts. Figure 2.35 shows a chromatogram and a spectrum for this irradiation with a peak whose retention time is 80.058 minutes. The UV spectrum for this peak is similar to the spectrum of PABA that has not been irradiated, i.e. two absorption maxima. This is consistent with literature findings that the absorption spectrum of PABA remains the same after long intense irradiation although a small fraction undergoes photochemical change **[235]** and that the UV spectra of PABA and its photoproducts are very similar in terms of shape and peak absorbance **[236]**. Gassparo *et al.* **[236]** reported the presence of *cis*- and *trans*-diazobenzoic acid in irradiated solutions of PABA that were oxygen saturated. Shaw *et al.* **[113]**. got the same photoproducts, besides other photoproducts on UV-irradiation of an oxygenated solution of PABA. On UV irradiation of a de-oxygenated solution they, however, observed only one photoproduct, i.e. of 4-(4′-aminophenyl)aminobenzoic acid, the presence of which was confirmed by NMR, electron impact mass spectroscopy and synthesis. They also found that the photoreaction of PABA depends on pH as much as it depends on the presence or absence of oxygen. If aerated solutions discolour to brown then the photoproduct is not 4-(4′-aminophenyl)aminobenzoic acid. The solution did not show this phenomenon hence the peak could represent unreacted PABA or 4-

(4'-aminophenyl)aminobenzoic. On the basis of the findings by Shaw *et al.* **[113]** at pH 3 the UV spectrum of the latter in water should show two λ_{max} at 322 nm and 226 and two λ_{min} at 261 nm and 217 nm. The spectrum obtained here shows λ_{max} at around 222 nm and 284 nm at pH 3 which is consistent with the UV spectrum of PABA. Unirradiated PABA was also analysed by HPLC in order to compare its retention time and the retention time of irradiated sample. For the same chromatographic conditions, the retention time was found to be 79.65 minutes. This agrees, within experimental error, with the retention time of 80.06 minutes for irradiated PABA. Hence, we can conclude that even on long UV irradiation of PABA at pH 3.0 no photoproducts were formed and the peak represents unreacted PABA. 4-(4'-Aminophenyl) aminobenzoic acid might have been formed in amounts below the detection limit of the PDA since its quantum yield for PABA photolysis is less than 0.0001 at low pH **[113]**. It might also have photodecomposed to a very small concentration of products due to the long period of irradiation.

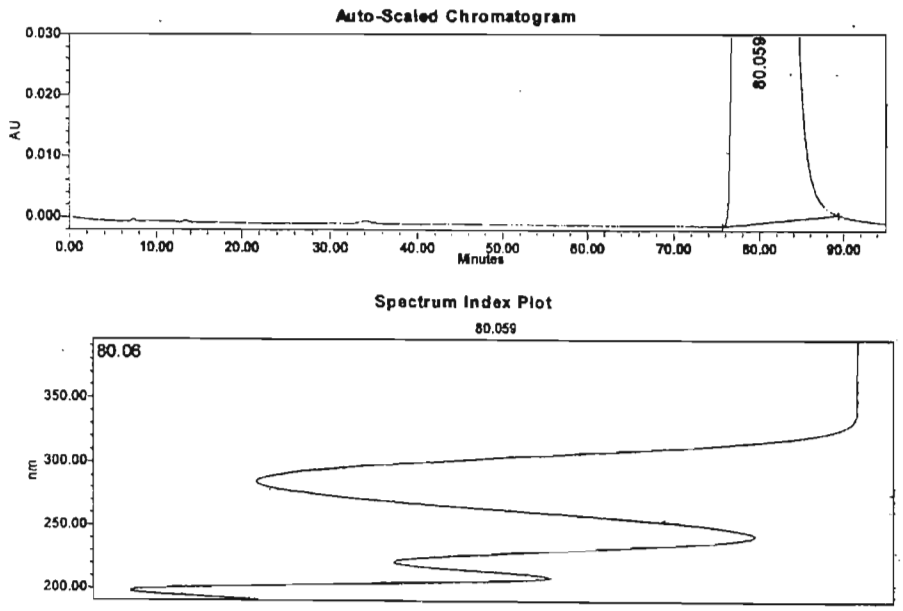

Figure 2.35: HPLC chromatogram of [PABA] = 1 x 10^{-3} M solution at pH 3.0 irradiated for 24 hours and eluted with a 4% (v/v) methanol mobile phase on an Ultracarb 5 ODS (20) column at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

From the irradiations of thymine and PABA, we can confidently conclude that photoproducts observed in our experiments are mainly from photochemical interactions between the two.

Irradiation of vitamin E alone gave only one UV-detectable photoproduct as seen in Figure 2.36. The UV-spectrum is quite different from that of un-irradiated vitamin E at pH 3.0 Irradiation of vitamin E thus produced a photoproduct with different absorption properties from itself. This photoproduct has a maximum UV absorption at around 205 nm whereas unirradiated vitamin E absorbs around 290 nm as shown in Figure 2.32. Vitamin E is known to undergo rapid photooxidation by UVB irradiation giving α-tocopherol dihydroxy dimer **[237]** (that results from reaction of two tocopheroxyl radicals) as the major photoproduct besides other minor ones.

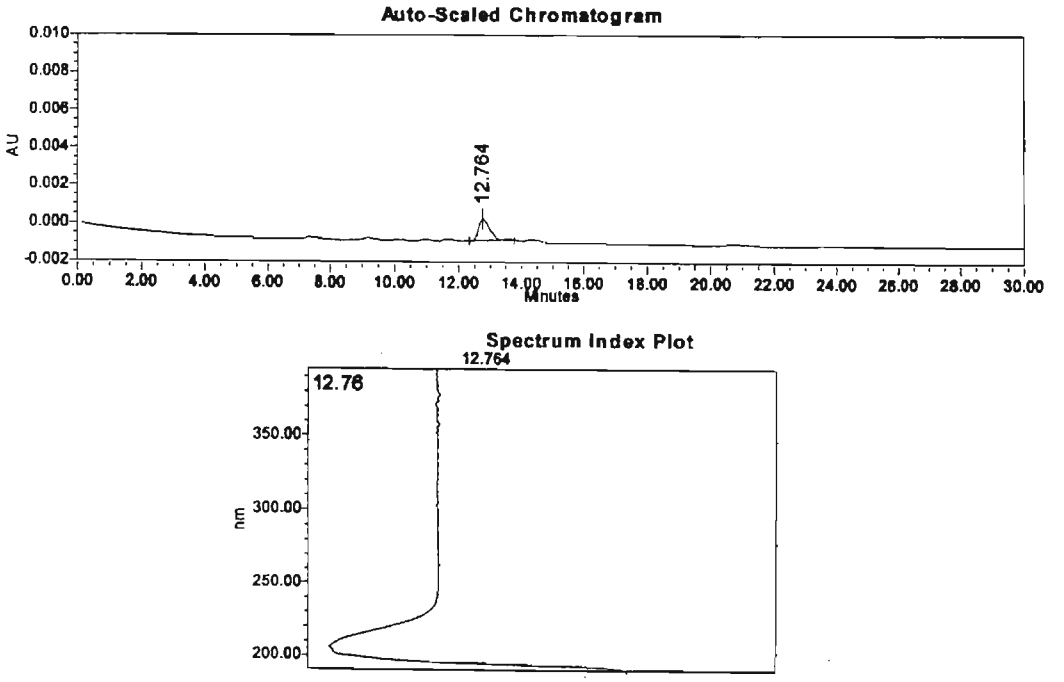

Figure 2.36: HPLC chromatogram and UV absorption spectrum for a 24-hours irradiation of [vitamin E] = 0.018 g L^{-1} solution at pH 3.0 It was eluted on an Ultracarb 5 ODS (20) column with a 4% (v/v) methanol mobile phase at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

There is only one detectable product shown in the chromatogram whereas the literature reports more than one **[237]**. It is possible that the amounts formed were too small to detect or the products photodecomposed due to the long hours of irradiation. These photoproducts are thought to be responsible for the protective effect of vitamin E **[238, 239]**.

Photoproducts resulting from irradiation of vitamin C are shown in the chromatogram in Figure 2.37. The peak at 12.59 minutes is for unreacted vitamin C whereas the other peaks represent photoproducts from the interaction of vitamin C with UV radiation. They could be products of photooxidation of ascorbate radical that were formed during irradiation and HPLC analyses **[183]**, although this is less probable since the solutions were deoxygenated prior to irradiation. This implies that UV radiation of vitamin C solutions can produce some photoproducts.

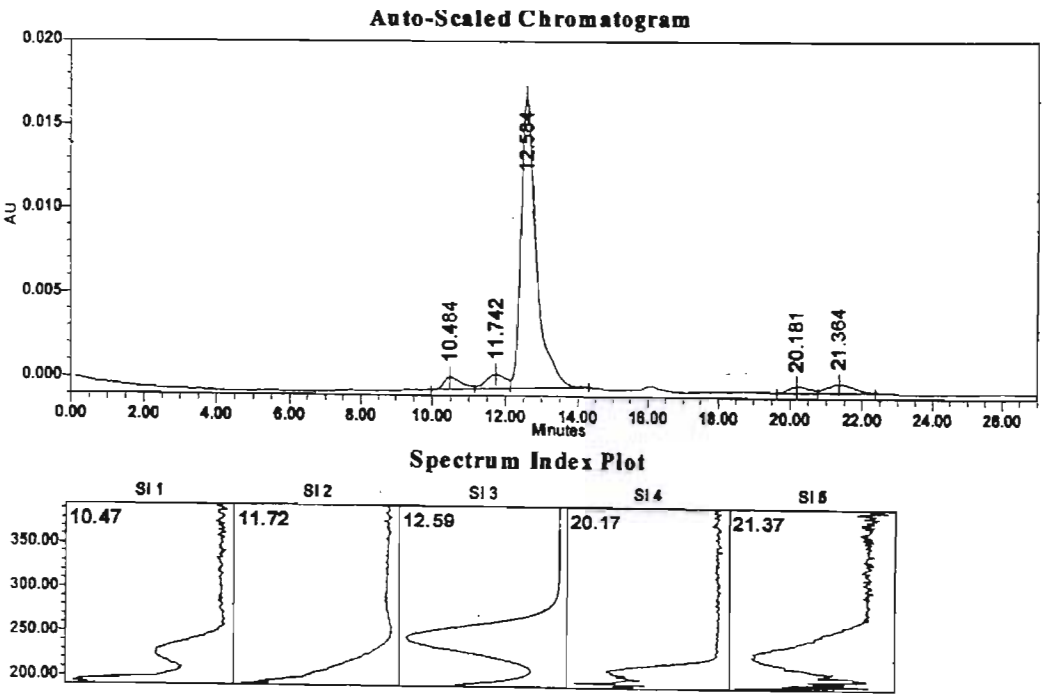

Figure 2.37: HPLC chromatogram of [vitamin C] = 0.044 g L^{-1} solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on an Ultracarb 5 ODS (20) column with a 4% (v/v) methanol mobile phase, at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

Photoproducts from the irradiation of thymine and vitamin C are shown in Figure 2.38. The presence of some dimer is evident from the peaks at 10.535 and 11.384 minutes, which represent *cis-anti* and *trans-anti* dimers, respectively. These were determined by the position of the dimers for the same chromatographic conditions. For these chromatographic conditions, *trans-syn* and *cis-syn* dimers should appear around 14.5 and 26 minutes respectively. The spectra obtained do suggest the presence of any dimer. The peaks at 11.9, 13.59 and 15.04 minutes are photoproducts that are likely to have originated from thymine.

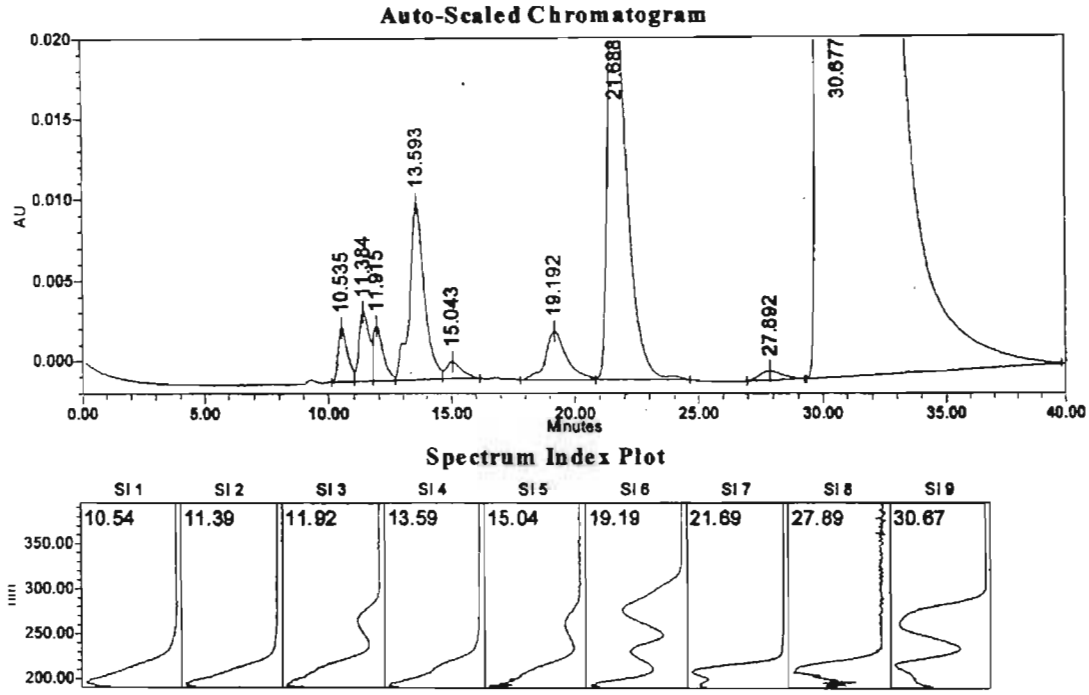

Figure 2.38: HPLC chromatogram of [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on Ultracarb 5 ODS column with a 4% (v/v) methanol mobile phase at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

The peaks at 13.59 and 15.04 minutes are the same components as the ones represented by 13.71 and 14.97 minutes respectively on irradiation of thymine alone (Figure 2.34). Therefore these two peaks can be attributed to the presence of thymine. The peak at 15.04 minutes has a maximum absorption at about 265 nm. These could be one of the one of the four photoproducts observed on irradiation of thymine alone. There is a photoproduct that elutes at about 19

minutes that is also seen on irradiation of pure thymine, thymine and vitamin C, and lastly thymine and vitamin E. It does not appear in the irradiation of pure vitamin C, nor pure vitamin E, nor in pure PABA. This implies that it is produced as a result of direct interaction of thymine with light to the exclusion of the other substrates. Compared to Figure 2.37, the peak for vitamin C at about 12.6 minutes is conspicuously missing despite the amount of vitamin C used being the same. The only plausible reason is that it could have lowered thymine dimer formation. The photoproduct at 21.688 minutes is absent in photoproducts of other blank samples except in the irradiation of thymine and vitamin E. The photoproduct at 27.892 minutes is absent in the photoproducts from irradiation of thymine and vitamin E (Figure 2.39). It is also absent in individual blank samples of thymine, vitamin C and PABA. Therefore, it could have arisen as a result of photochemical interaction between thymine and vitamin C. The peak at 30.677 minutes is for thymine.

Photoproducts from irradiation of pure thymine and pure vitamin E are shown in Figure 2.39. Some dimers are formed as evident from peaks at 10.485, 11.302, and 23.884 minutes. They represent *cis-anti, trans-anti,* and *cis-syn* respectively. The UV spectrum for the peak at 13.528 is similar to that of dimer and appears close to where *trans-syn* thymine dimer appears for these irradiation conditions. This could be the dimer or an unknown photoproduct from thymine. There was no standard for this dimer hence it is difficult to clearly identify it is a dimer or an unknown photoproduct. Since irradiated vitamin E gives only one peak at 12.76 minutes, that cannot be seen here, these dimers and photoproducts could be due to the presence of both thymine and vitamin E. The peaks at 14.932 and 18.839 minutes are photoproducts that are also observed on irradiation of pure thymine but are not observed on irradiation of blanks of PABA or vitamin E. The peak at 14.932 is not seen on addition of vitamin C but the peak at 18.468 minutes is similar to the peak at 19.19 minutes in Figure 2.38. Their presence can only be attributed to thymine. The peak at 21.468 minutes is not observed on irradiation of pure thymine but is observed in all other systems in which thymine is involved. Therefore, this photoproduct is formed from interaction of thymine with radiation and another substrate.

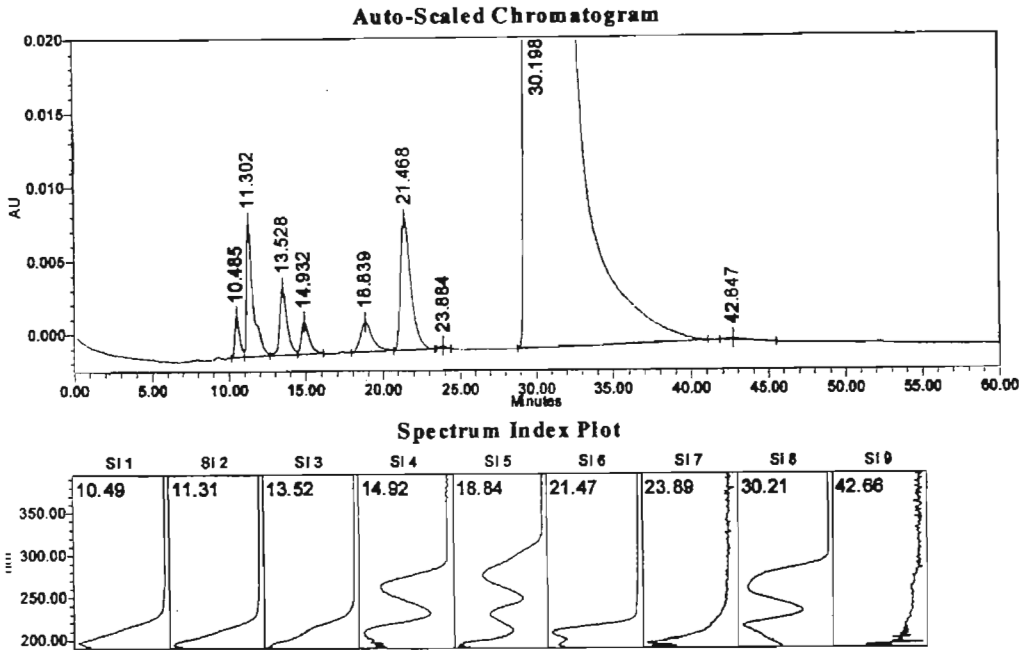

Figure 2.39: HPLC chromatogram of [vitamin E] = 0.018 g L^{-1} and [thymine] = 1 x 10^{-2} M solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on an Ultracarb 5 ODS column with mobile phase of a 4% (v/v) methanol at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

Photoproducts resulting from the irradiation of vitamin C and PABA are shown in Figure 2.40. In this figure, there is no peak in the chromatogram indicating the presence of vitamin C despite the same amount having been used as when vitamin C was irradiated alone (See Figure 2.37). While using riboflavin as a photosensitiser, Kitazawa *et al.* **[177]** showed that it could reduce the antioxidant activity of vitamin C by reacting with it. We cannot rule out a similar phenomenon with regard to PABA, which is also a photosensitiser. The peak at 68.982 minutes results from irradiation of PABA because its spectrum corresponds to that of PABA with a retention time of 80.059 minutes in Figure 2.35. After about 35 minutes of elution of the components, the mobile phase was changed to 100% methanol (v/v) so as to hasten the elution of any photoproducts due the presence of PABA. The peak at 11.387 minutes although very small, is also found in the chromatogram of irradiated vitamin C alone. The rest of the peaks are found in neither the chromatogram of PABA nor in that of vitamin C. This indicates that they were formed from the interaction of

vitamin C and PABA. However, apart from the product at 68.96 minutes the other photoproducts are formed in virtually undetectable amounts.

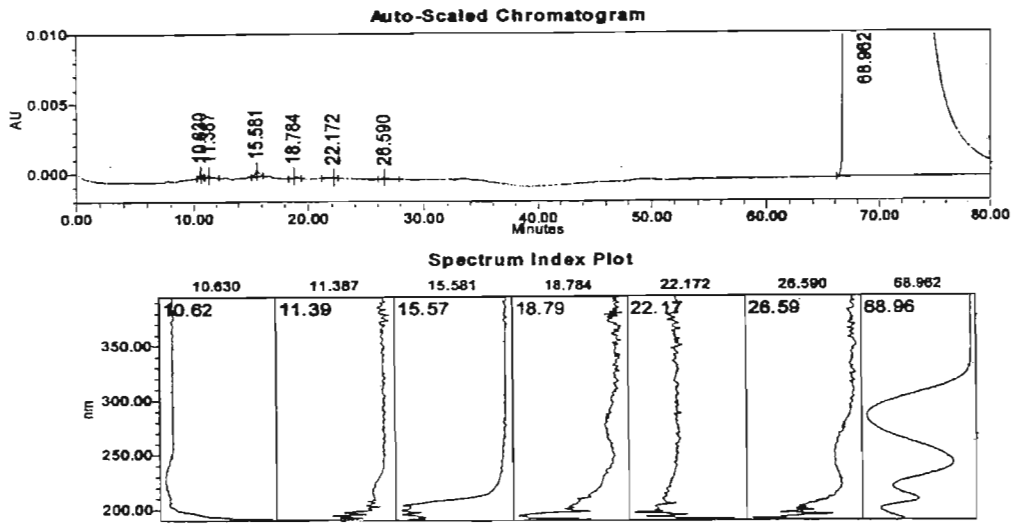

Figure 2.40: HPLC chromatogram of [vitamin C] = 0.044 g L^{-1} and [PABA] = 1 x 10^{-3} M solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on Ultracarb 5 ODS (20) column with a 4% (v/v) methanol mobile phase at a flow rate of 1.5 mL min^{-1} then 100% methanol to hasten elution of PABA and detected at 220 nm.

Photoproducts of the irradiation of vitamin E and PABA are shown in Figure 2.41. The peak at 72.605 minutes represents PABA as seen in Figure 2.35 where the retention time for PABA is 80.059 minutes. The times for PABA in Figures 2.40 and 2.43 are different because the mobile phase has also changed. Despite differences in retention times, the spectrum for the peak at 13.802 minutes is similar to the spectrum at 12.764 minutes in Figure 2.36, i.e. due to irradiation of vitamin E alone. This strongly suggests that it results from the presence of vitamin E. The peak at 15.953 minutes is not present in the chromatogram for the irradiation of vitamin E nor the chromatogram of PABA. This likely resulted from the photochemical reaction of PABA and vitamin E.

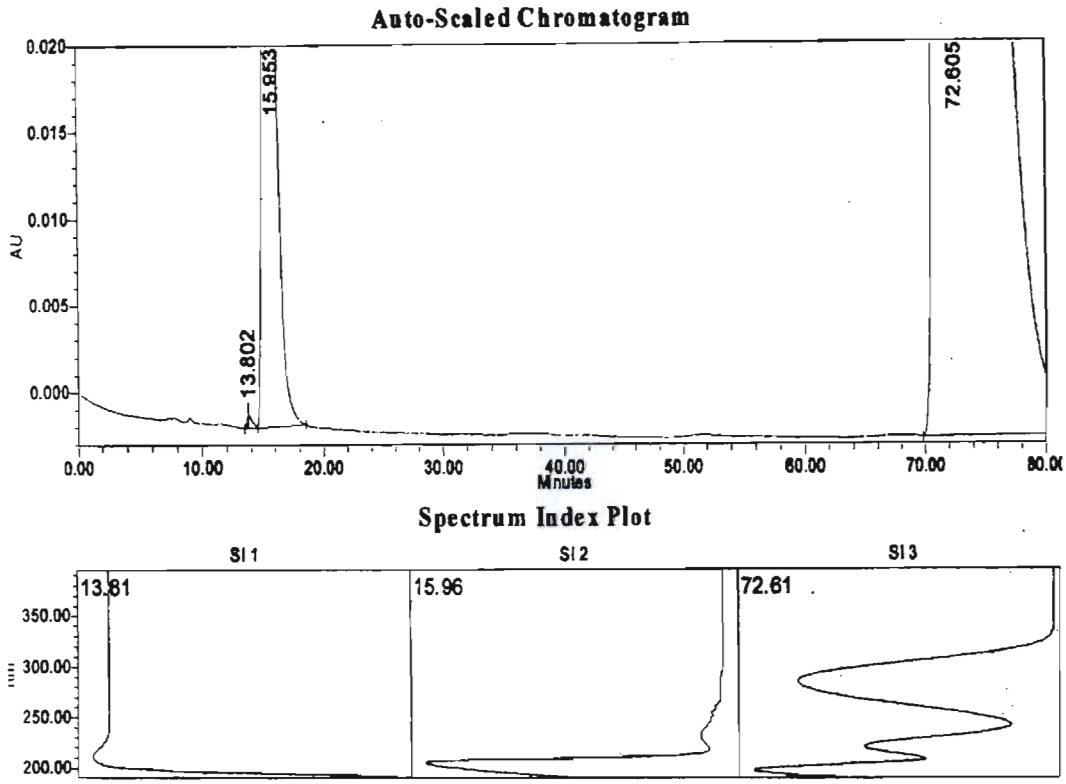

Figure 2.41: HPLC chromatogram of [vitamin E] = 0.018 g L^{-1} and [PABA] = 1 x 10^{-3} M solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on an Ultracarb 5 ODS (20) column with a 4% (v/v) methanol mobile phase of at a flow rate 1.5 mL min^{-1}. After 35 minutes the mobile phase was changed to 100% methanol in order to hasten elution of PABA. The peaks were detected at 220 nm.

Photoproducts from the irradiation of pure vitamin C and vitamin E are shown in Figure 2.42. No peak is observed at about 12.76 minutes representing irradiated vitamin E. A peak for unreacted vitamin C would have been observed at around 12 minutes for unreacted but none is present. Vitamin C is known to be consumed by UV radiation. In the presence of tocopheryl radical, the consumption is more rapid since vitamin C regenerates vitamin E from the radical **[177, 183]**. The photoproduct(s) seen in the irradiation of pure vitamin E and pure vitamin C are not present in this chromatogram.

Figure 2.43 shows photoproducts from the irradiation of thymine, vitamin E and vitamin C together. There is no peak present for unreacted vitamin C. All

vitamin C must have been destroyed or consumed to regenerate vitamin E that must also have been destroyed by UV radiation **[177]**. The peak at 12.764 minutes in Figure 2.36 for the photoproduct formed on irradiation of vitamin E alone is also not observed. The peaks at 10.066 and 35.108 minutes are *cis-anti* and *cis-syn* thymine dimers respectively. Because of change in composition of the mobile phase, the retention times for these dimers changed from around 12 minutes and 37 minutes respectively. No trace of *trans-syn* dimer was observed whereas *trans-anti* dimer might have formed but is not resolved from thymine or might not have been formed at all. The peaks for these two dimers if present would have been expected to appear at about 18 and 46 minutes respectively. The peaks at 18.077, 21.805 and 23.915 minutes can be seen only in the chromatogram of the irradiation that includes thymine alone. The UV-spectra in Figure 2.34 show very weak absorption of these photoproducts. The products are therefore certainly from interaction of thymine with radiation.

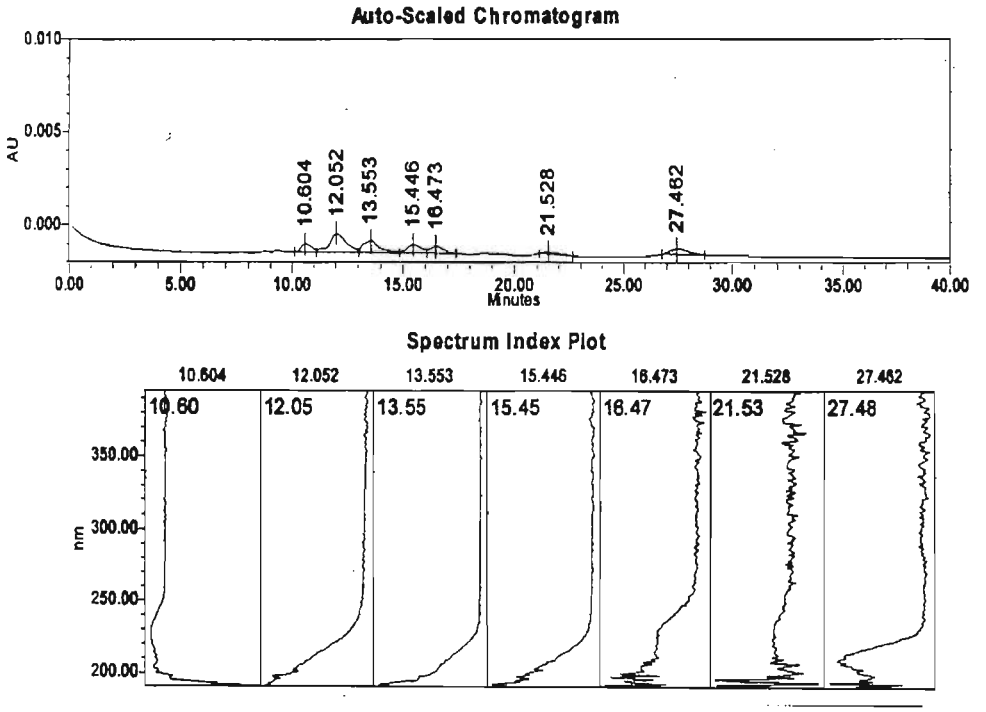

Figure 2.42: HPLC chromatogram of [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on an Ultracarb 5 ODS (20) column with a 4% (v/v) methanol mobile phase, at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

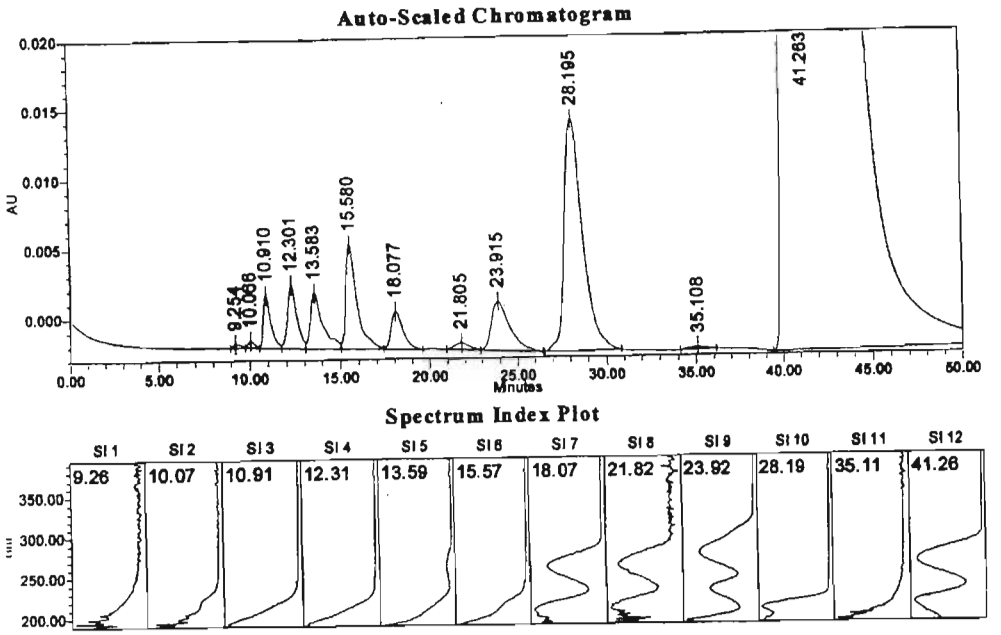

Figure 2.43: HPLC chromatogram of [thymine] = 1 x 10^{-2} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on an Ultracarb 5 ODS (20) column with a 1% (v/v) methanol mobile phase, at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

Photoproducts of irradiation of vitamin C, vitamin E and PABA are shown in Figure 2.44. No vitamin C is seen at all in the chromatogram implying that it was either consumed or whatever might not have reacted was destroyed by the long period of irradiation. The absence of vitamin C in this chromatogram is not surprising because of the presence of both vitamin E and PABA. Kitazawa *et al.* [177] showed that vitamin C is photolysed by UV radiation and is rapidly consumed in the presence of vitamin E radical to regenerate vitamin E. While using riboflavin, a triplet photosensitiser, they also showed that the consumption is accelerated by the presence of a photosensitiser. Therefore, in our experiments, we would expect the presence of PABA to have a similar effect as riboflavin. The peak at 11.267, 13.808, 20.995 and 67.705 minutes also appear in the blanks for PABA, vitamin C, vitamin E and thymine, respectively. The peaks at 9.225, 10.540 and 25.874 minutes are photoproducts that are not observed in the controls. The absorption HPLC peak area for these

photoproducts is very weak indicative of the fact that they are formed in very small quantities.

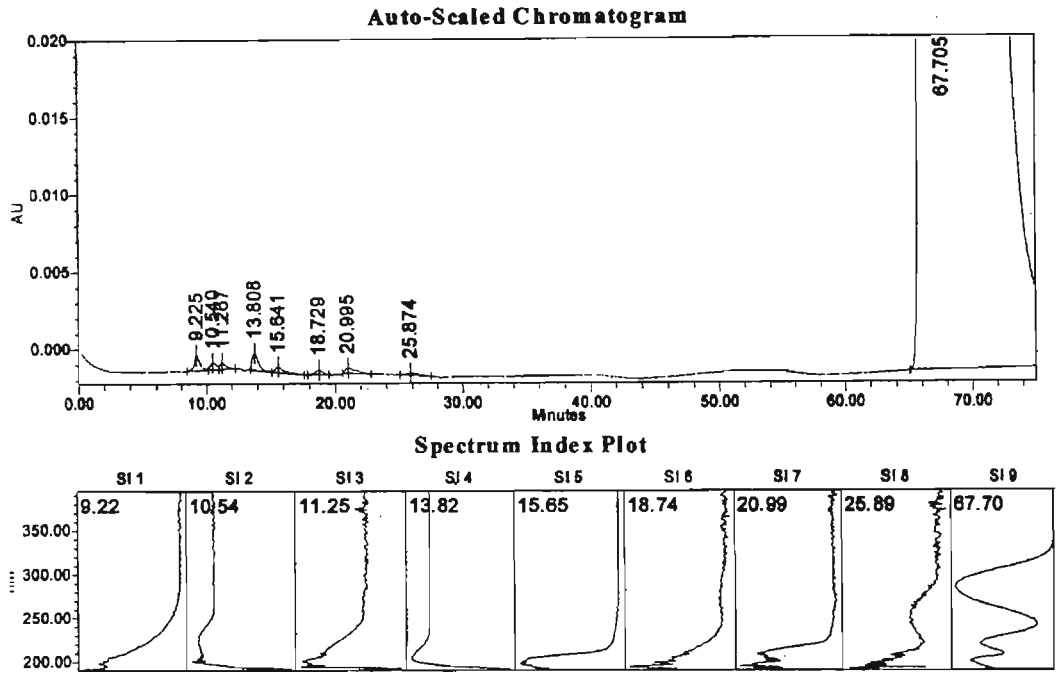

Figure 2.44: HPLC chromatogram [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [PABA] = 1 x 10^{-3} M solution at pH 3.0 irradiated for 24 hours. The photoproducts were resolved on Ultracarb 5 ODS (20) column with a 4% (v/v) methanol mobile, at a flow rate of 1.5 mL min^{-1} and detected at 220 nm.

From the preceding paragraphs it is evident from experiments involving controls that irradiated solutions that contained thymine can also form dimers in the absence of a photosensitiser. Besides the four thymine dimer isomers, other photoproducts are also produced. From the thymine concentration used in the irradiation, i.e. 1 x 10^{-2} M, there are two plausible mechanisms. Either diffusion-controlled dimerisation between ground state thymine and the excited triplet state or dimerisation from an aggregate. Although there was enough thymine, dimerisation between ground state thymine and its singlet is unlikely because of rapid decay of the singlet in 10^{-11} seconds **[34, 80]**. Other than the thymine dimers, thymine monomer and PABA, other photoproducts were not identified. These photoproducts appearing on the chromatograms involving thymine could possibly be uracil, 5-hydroxymethyluracil and 5-formyl uracil

[240]. The identities of the photoproducts were not verified because the amounts formed were too small. It is also evident that interaction of UV radiation with the vitamins, or thymine and vitamins, or PABA and vitamins yielded some photoproducts that are not formed in the typical thymine and PABA irradiation. Vitamin C is consumed more rapidly by UV radiation than vitamin E. Since there was base resolution between all the peaks, the presence of these photoproducts did not affect quantitation of the dimers.

2.8.4 EXPERIMENTAL CONDITIONS

The concentrations and irradiation times used to quantify the PABA-photosensitised dimerisation of thymine are tabulated in Tables 2.13 to Table 2.17.

Table 2.13: Concentrations used for experiments in which the irradiation time was varied for a mixture of thymine, PABA, vitamin E and vitamin C at pH 3.0

[Thymine]/ M	[PABA]/ M	[Vitamin E]/ g L^{-1}	[Vitamin C]/ g L^{-1}	Irradiation/ Hours
1×10^{-2}	1×10^{-3}	0	0	1 - 19
1×10^{-2}	1×10^{-3}	0.018	0	5 - 24
1×10^{-2}	1×10^{-3}	0	0.044	5 - 24
1×10^{-2}	1×10^{-3}	0.018	0.044	5 - 24

Table 2.14: Concentrations used for experiments in which a mixture of 1×10^{-2} M thymine, PABA, vitamin E and vitamin C at pH 3.0 was irradiated for 6 hours whilst varying PABA concentration. The vitamins were added individually and also together. The concentrations were [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

[PABA]/10^{-3} M	[PABA]/10^{-3} M	[PABA]/10^{-3} M
0.8	4	12
1	8	15
2	10	20

Table 2.15: Concentrations used for experiments in which a mixture of 1 x 10^{-3} M PABA, vitamin E and vitamin C at pH 3.0 was irradiated for 6 hours whilst varying thymine concentration. The vitamins were added individually and also together. The concentrations were [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

[Thymine]/1 x 10^{-3} M	[Thymine]/1 x 10^{-3} M
1	6
2	7
3	8
4	9
5	10

Table 2.16: Concentrations used for experiments in which a mixture of 1 x 10^{-3} M of PABA, 1 x 10^{-2} M of thymine and vitamin E at pH 3.0 was irradiated for 6 hours whilst varying vitamin E concentration. Vitamins C of 0.044 g L^{-1} was added.

[Vitamin E]/g L^{-1}	[Vitamin E]/g L^{-1}
0.003	0.018
0.006	0.021
0.009	0.024
0.012	0.030
0.015	

Table 2.17: Concentrations used for the experiments in which a mixture of 1 x 10^{-3} M of PABA, 1 x 10^{-2} M of thymine and vitamin E at pH 3.0 was irradiated for 6 hours whilst varying vitamin C concentration. Vitamins C of 0.018 g L^{-1} was added.

[Vitamin C]/g L^{-1}	[Vitamin C]/g L^{-1}
0.010	0.076
0.022	0.088
0.044	0.098
0.066	0.108

The data obtained from these sets of experiments are presented in Appendix C. They have been plotted to show trends in dimer yield. Each graph presents a set of data in which only one variable (concentration of one component or irradiation time) was varied. The trends in the dimer yield are discussed in Chapter 3.

2.9 PABA-PHOTOSENSITISED SINGLET OXYGEN STUDIES

Previously reported studies of singlet oxygen involve mainly its generation, detection and measurement **[241]**. Laser pulse techniques and photosensitisation reactions are the main sources of singlet oxygen. These methods involve energy transfer from an excited triplet photosensitiser to ground state oxygen. Different methods have been used to detect and measure singlet oxygen. Among these methods are: time-resolved thermal lensing **[241]**, photoacoustic calorimetry **[242]**, photochemistry **[243]** and near-IR luminescence **[244, 245]**. Detection by photochemical methods is popular because it is simple, sensitive and cheap. It involves a photosensitiser and a probe for singlet oxygen. Some of the commonly used photosensitisers are methylene blue and Rose Bengal. Examples of acceptors are the azide ion, imidazole, and 1,3-diphenylisobenzofuran. The choice of photosensitiser and probe depends on their absorption maxima and solubilities in given solvents **[88, 89, 246]**. The absorption of the photosensitiser has to be in the

region where the probe is almost transparent **[246]**. The type of solvent also determines the quantum yield of singlet oxygen **[247]**. The lifetime of singlet oxygen is shortest in water. The rate of decay of singlet oxygen is solvent dependent. In aqueous solution, there are competing quenching reactions for singlet oxygen. This results in emission of singlet oxygen that is weaker than 1269 nm (its characteristic emission) and hence making it very difficult to detect **[90]**. Its formation can only be inferred from interception (scavenging) or quenching **[113, 248]**.

Sensitisers that have their lowest triplet state above 13 100 cm^{-1} can produce both $^1\Sigma_g$ and $^1\Delta_g$ oxygen **[249]**. The triplet excited state of PABA has been reported as 313.9 kJ mol^{-1} **[99]**. Thus, this excited triplet state of PABA clearly has enough energy to promote ground state oxygen to $^1\Delta_g$ oxygen. PABA is known to photosensitise the formation of singlet oxygen in aqueous solution **[120]**. In this work PABA was used to photosensitise the generation of singlet oxygen. Vitamin E and vitamin C are known to react with free radicals and scavenge singlet oxygen. This section of the experimental work was carried out in order to investigate photosensitised formation of singlet oxygen by PABA in the presence of the two antioxidants by using the photochemical method developed by Kraljic *et al.* **[248]** for the detection of singlet oxygen. This method is described in the next section.

2.9.1 DETECTION OF SINGLET OXYGEN

Kraljic *et al.* **[248]** have developed a method for detecting and measuring singlet oxygen in aqueous solutions. The formation of singlet oxygen can be followed by use of N,N-dimethyl-4-nitrosoaniline (RNO) as a selective scavenger. A singlet oxygen acceptor, A (like histidine or imidazole) induces bleaching of RNO to give a bleached RNO, (*RNO). This happens through a *trans*-annular peroxide intermediate, $[AO_2]$, which is capable of inducing the bleaching. This is shown in the following reaction scheme:

$$^1O_2 + A \rightarrow [AO_2] \rightarrow AO_2$$

$$[AO_2] + RNO \rightarrow *RNO + Products$$

Because RNO has a large molar absorption coefficient, the bleaching can be followed spectrophotometrically at 440 nm for high pH values or at 350 nm for low pH values (absorption maxima of RNO). This method is very applicable for an air-saturated solution. In the absence of RNO, the intermediate decomposes or rearranges into oxygenated products **[113, 248, 250, 251]**. Physical quenching of a triplet photosensitiser by RNO can be considered as negligible at the concentrations normally used for RNO, namely 40-50 μM **[252]**. At these concentrations, RNO scavenges the secondary reactive intermediates and thus protects the photosensitiser **[252]**. Therefore a combination of PABA and RNO would be appropriate in our investigation.

2.9.2 CHOICE OF EXPERIMENTAL CONDITIONS

In singlet oxygen studies, the irradiation time, the choice of acceptor, and the concentrations of RNO, photosensitiser and other substrates involved, are crucial. The method has to be sensitive and able to cause produce a measurable change in absorbance. The concentrations of the species have to be such that they do not react with other intermediates formed in the system **[248]**. Long irradiation times that will bleach more than 10 to 15% of RNO are also undesirable **[248]**. The following paragraphs detail the experiments performed to determine the best acceptor and the optimal concentrations and irradiation times.

In order to reduce or eliminate secondary reactions on RNO, it is preferable to carry out experiments at very low concentrations of RNO and other species. At lower concentrations, RNO is stable in the presence of other oxidizing species besides singlet oxygen and can therefore be used to obtain data for singlet oxygen studies **[251]**. At concentrations of RNO higher than 0.1 M, the reaction between the triplet photosensitiser and the substrate may predominate

[253]. Many studies have shown that the optimal concentration for RNO is 4 x 10^{-5} M for different systems studied **[250, 251, 254, 255]**.

Initially, histidine was used as the acceptor to give the *trans*-annular complex that reacts with RNO. After several trials it was abandoned because it did not give an appreciable change in absorbance. Kraljic *et al.* **[248]** tried the system imidazole/RNO and found it to produce greater changes in the concentration of RNO than the system histidine/RNO **[248]**. Imidazole was used in place of histidine. It gave an appreciable change in absorbance and hence, was used in this experimental work. A phosphate buffer of pH 3.0 was used to maintain the pH of the sample solution during the investigation. This was necessary because the wavelength of maximum absorption of RNO changes with pH.

No studies have been previously reported using RNO, PABA and imidazole at pH 3.0. Most studies on singlet oxygen measurements using RNO have been done at pH 7 or higher. Substrates that absorb light of wavelengths greater than 300 nm are not suitable. Those that chemically react with any of the substrates are also not suitable. Therefore, preliminary irradiations of solutions containing all the substrates were carried out and their UV spectra were measured to ascertain their suitability in our investigation at pH 3.0

UV spectra for aqueous solutions at pH 3.0 of all the species involved, i.e. the buffer, were acquired. Figure 2.45 shows the UV spectra for PABA, imidazole, thymine dimer, thymine monomer, RNO, the two vitamins and the buffer. The buffer was used as the solvent for all sample preparations. Superimposed on these spectra is the transmission spectrum of the 10 mm thick Pyrex filter in order to determine which of the reagents absorbs radiation of wavelength greater than 300 nm. From the spectra it is evident that only PABA, RNO and vitamin E absorb at the wavelengths of irradiation. From the spectra at pH 3, the absorption maxima for RNO, PABA, imidazole, and the phosphate buffer are 350 nm, 284 nm, 223 nm and 209 nm, respectively.

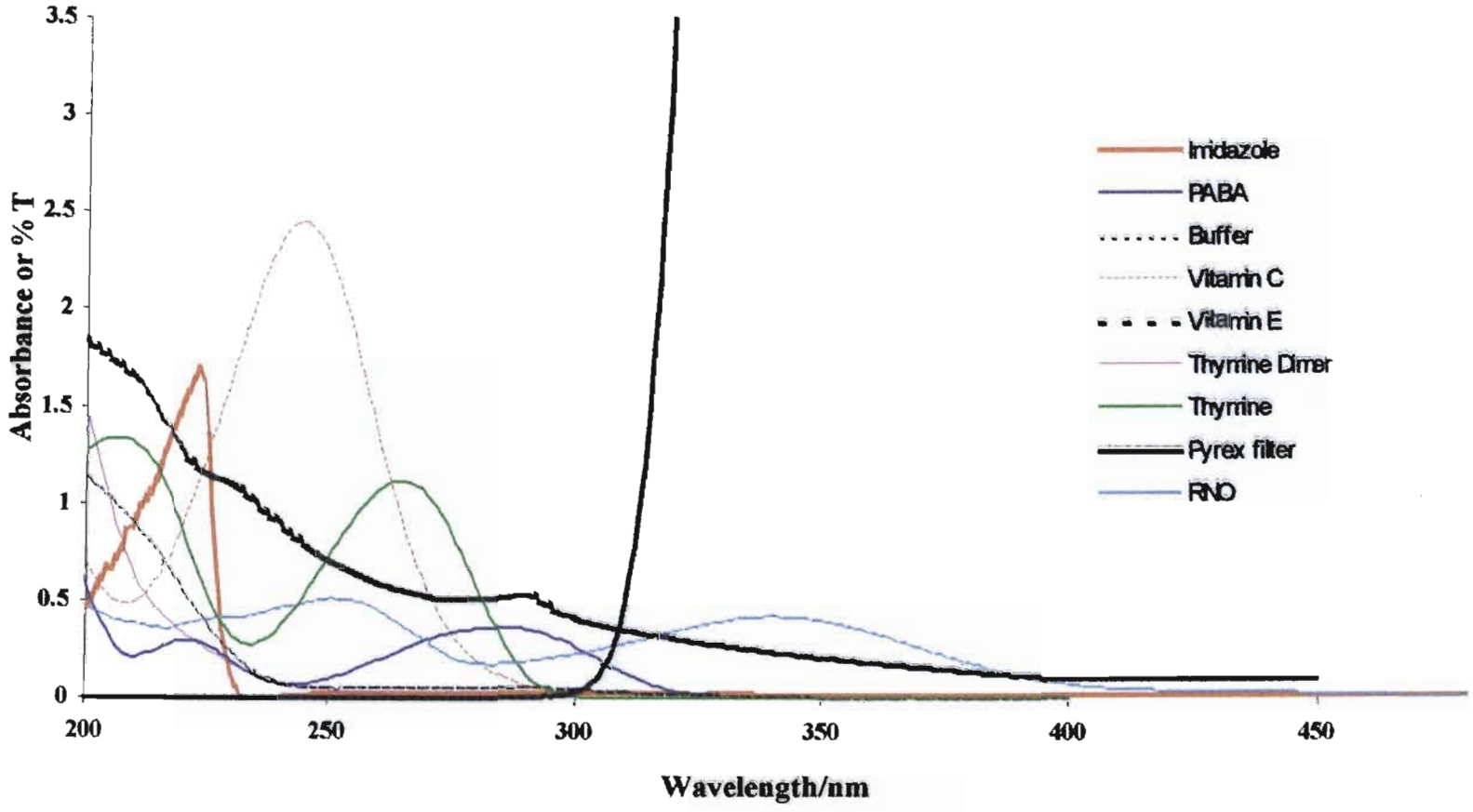

Figure 2.45: UV spectra of the buffer and aqueous solutions of reagents used in the investigation of the PABA-photosensitized formation of singlet oxygen in aqueous solution at pH 3.0 obtained in a 10 mm pathlength quartz cell. Superimposed on these spectra is the transmission spectrum of the 10 mm thick Pyrex filter used in the irradiations.

Figure 2.46 shows the UV spectra of RNO at pH 3.0 for an un-irradiated sample and for samples irradiated for varying lengths of time. The wavelength of maximum absorption is 350 nm. This agrees with studies by some researchers who found the absorption maximum shifts to 350 nm at lower pH. This is attributed to the pK_a for the acid-base equilibrium between the protonated and free RNO, which is 3.7. At high pH values the absorption is 440 nm **[250]**. It is also evident from Figure 2.46 that the absorption of RNO diminishes as the irradiation time is prolonged. This implies that the amount of RNO is depleted as the irradiation time is increased. The spectrum for irradiation of RNO for a period of one hour shows that the wavelength of maximum absorption has shifted to a lower value than 350 nm. This agrees with the findings of Kraljic *et al.* **[250]** that the absorption maximum shifts towards wavelengths shorter than 440 nm for prolonged irradiation. This shift in absorption is caused by photoproducts that have maximum absorption at 428 nm or lower **[250]**. From the spectra shown in Figure 2.45, it can be seen that the absorbance of RNO can be followed without interference of absorption from the rest of the components. In this work, the formation of singlet oxygen was therefore investigated by following the absorbance of RNO at 350 nm.

A mixture containing PABA of concentration 1×10^{-3} M, RNO of concentration 4×10^{-5} M, and varying imidazole concentrations was irradiated for six, twelve, fifteen and thirty minutes. This was carried out in order to find the best irradiation time that would produce a measurable change in absorbance and also find the optimum concentration for imidazole that could be used. Fifteen minutes produced an appreciable change in absorbance. Irradiation times longer than fifteen bleached RNO by more than 10 to 15%, which is undesirable when using it to monitor singlet oxygen changes **[248]**. Table 2.18 shows the change in absorbance obtained for varying imidazole concentrations when the mixture was irradiated for fifteen minutes.

A plot of change in absorbance of the samples at 350 nm against the logarithm of the imidazole concentration is shown in Figure 2.47. The maximum of the plot gave a value of 8×10^{-3} M of imidazole as the optimal value. This agrees very well with literature values obtained for imidazole and it is low enough not

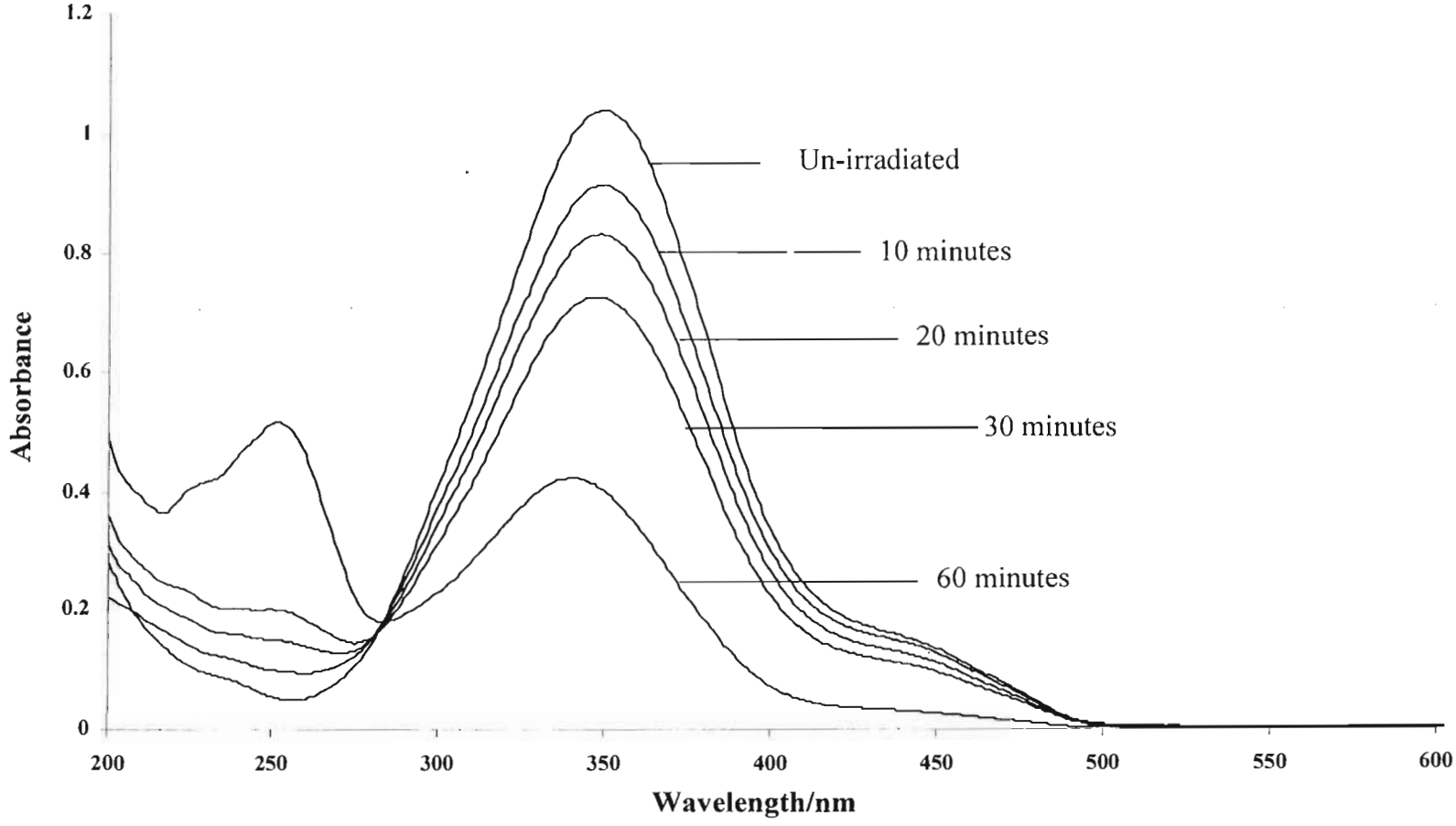

Figure 2.46: UV spectra of un-irradiated and irradiated solutions of [RNO] = 4.0 x 10^{-5} M in a phosphate buffer of pH 3.0 obtained in a 10 mm pathlength cuvette.

to cause secondary reactions **[248]**. Imidazole has a very low triplet quenching rate constant **[253]**. Therefore, at concentrations lower than 0.01 M, chemical reaction between imidazole and the triplet sensitiser in air-saturated solutions is negligible. Thus, the reaction proceeds via the trans-annular mechanism **[248, 253]**. An imidazole concentration of 8 x 10^{-3} M was thus used in these experiments.

Table 2.18: Change in absorbance at 350 nm observed with change in imidazole concentration when solutions at pH 3.0 containing imidazole, RNO = 4 x 10^{-5} M and [PABA] = 1 x 10^{-3} M were irradiated for 15 minutes with the Osram HBO 500 W high pressure mercury lamp and pyrex filter combination.

[Imidazole]/M	Change in Absorbance
0.0003	0.1532
0.001	0.2701
0.003	0.3451
0.01	0.3355
0.03	0.2668
0.1	0.1502
0.3	0.0013

It has been reported that on irradiation with UV radiation, a 4,5-subsituted imidazole reacts with thymine by photocycloaddition **[256]**. Preliminary experiments were performed to ensure that no secondary reactions occur under our experimental conditions. Therefore, the UV-spectrum of an un-irradiated mixture of imidazole and thymine at pH 3.0 was compared to that of an irradiated mixture. The mixture was irradiated for 20 minutes, the longest irradiation time used. Figure 2.48 shows the two spectra superimposed on one another. From the two spectra, there is no change in absorbance as a result of the irradiation. This implies that imidazole does not affect thymine on irradiation. Therefore imidazole can be used as an acceptor to investigate the effect of thymine in the formation of singlet oxygen.

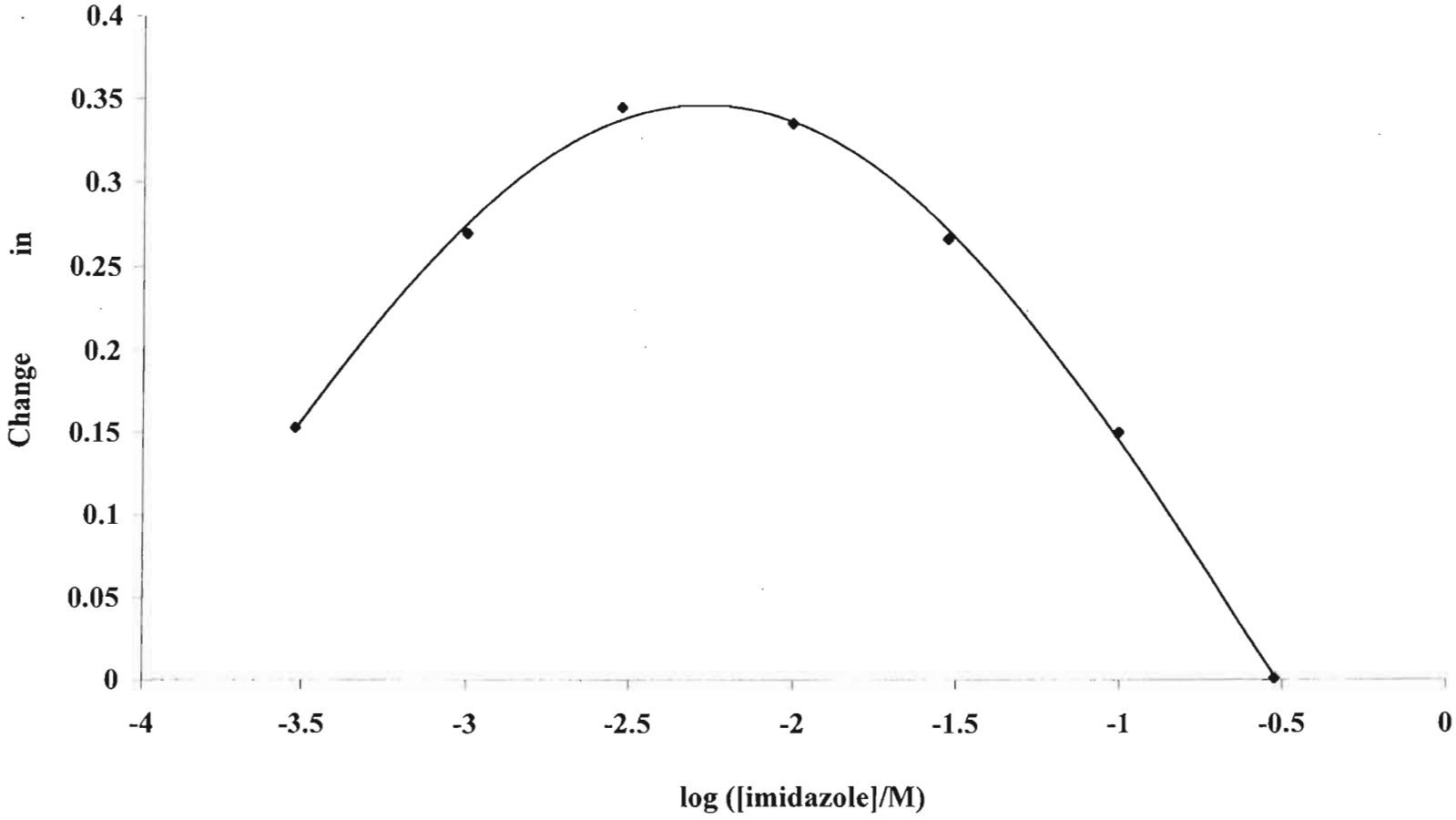

Figure 2.47: Change in absorbance at 350 nm for a mixture of [PABA] = 1 x 10^{-3} M, [RNO] = 4 x 10^{-5} M and variable amounts of imidazole at pH 3.0 for 15 minutes irradiation with the Osram HBO 500 W high pressure mercury lamp and pyrex filter combination.

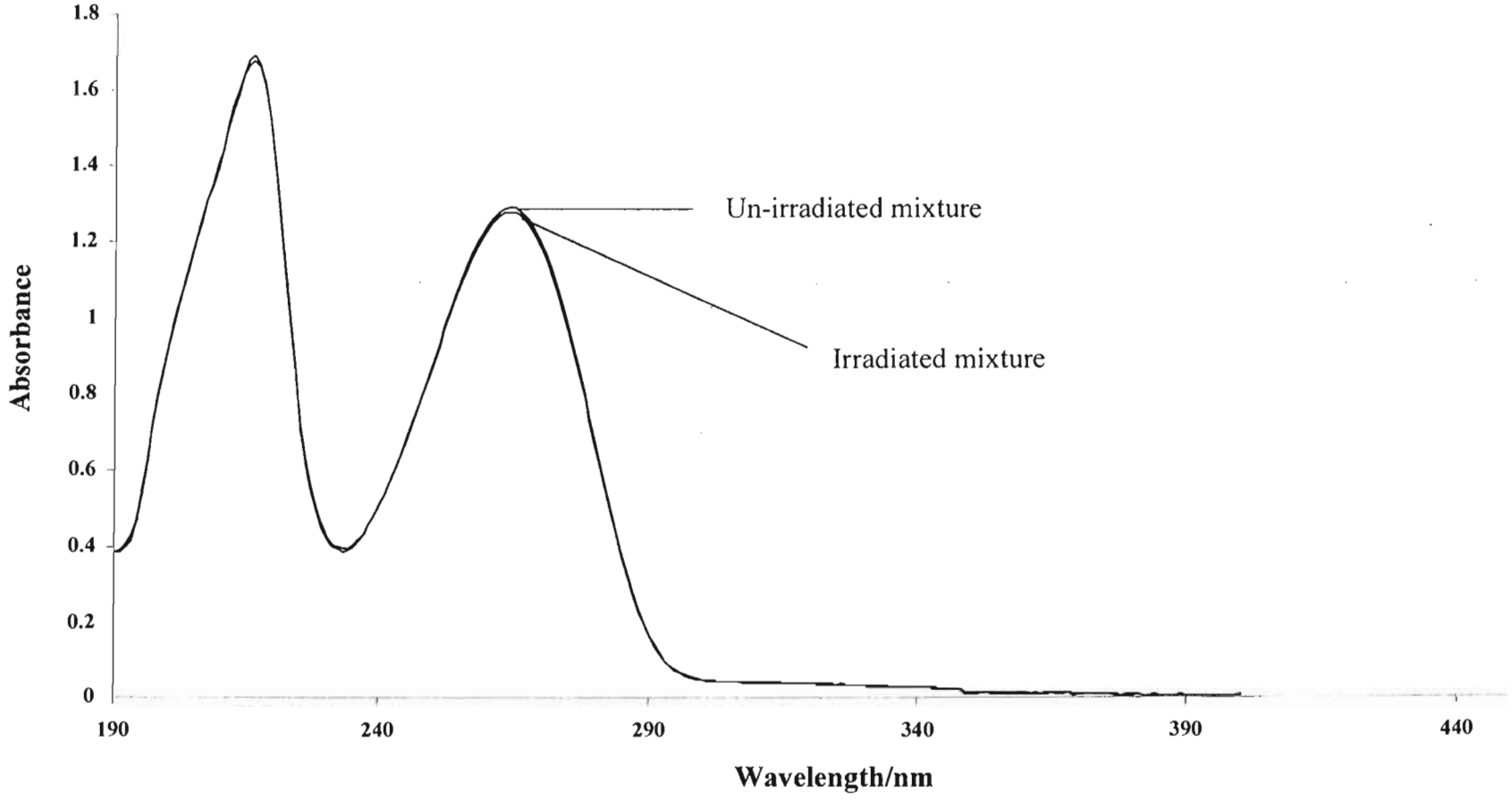

Figure 2.48: UV spectra of an irradiated and un-irradiated mixture of [thymine] = 6.5 x 10^{-3} M and [imidazole] = 1.6 x 10^{-3} M at pH 3.0 obtained in a 10 mm pathlength quartz cuvette.

In the thymine photosensitisation studies, the concentration of PABA was varied between 4 x 10^{-5} M and 2.0 x 10^{-2} M. A similar concentration range was tested and used for the singlet oxygen studies. A concentration range between 3.0 x 10^{-5} M and 1.5 x 10^{-2} M was used to determine whether PABA could photosensitise the formation of singlet oxygen. Table 2.19 shows the change in absorbance obtained on irradiation for 15 minutes of RNO, imidazole and varying concentrations of PABA. Figure 2.49 is a plot of the values shown in the Table 2.19. The linear plot obtained indicates that the concentration range used for PABA is sufficient to produce singlet oxygen that is detectable with the concentrations of RNO and imidazole used. This concentration range was used for PABA in our experiments. The concentrations of thymine, vitamin E and vitamin C were maintained at 1 x 10^{-2} M, 0.018 g L^{-1}, and 0.044 g L^{-1} respectively, i.e. the concentrations used in the photodimerisation studies.

Table 2.19: The change in absorbance monitored at 350 nm for 15 minutes irradiation with the Osram HBO 500 W high pressure mercury lamp in combination with 10 mm pathlength Pyrex filter of a mixture of [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and varying concentrations of PABA.

[PABA]/10^{-2} M	Change in Absorbance
1.5	0.2011
1.2	0.1885
0.9	0.1798
0.6	0.1501
0.3	0.1274
0.15	0.1050
0.015	0.1052
0.003	0.1029

Figure 2.50 presents the UV-spectra showing the effect of irradiation time on pure RNO and on a mixture of RNO, PABA and imidazole. It is evident that at long irradiation times the absorbance at 350 nm changes significantly. Irradiation times used in the thymine photodimerisation experiments could not be used in the singlet oxygen studies. During the investigation of thymine photodimerisation, the shortest irradiation time was one hour. This time is

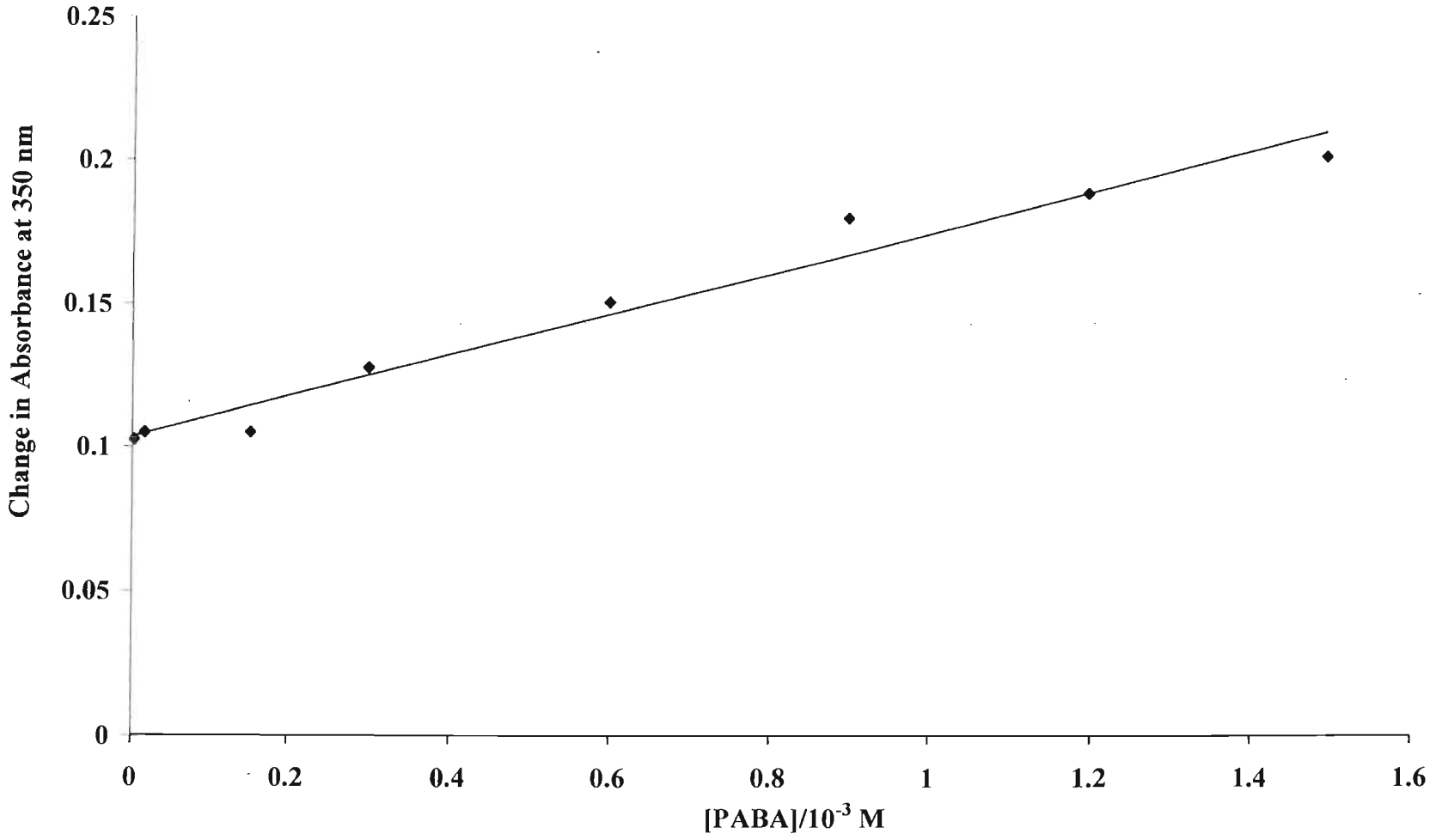

Figure 2.49: Change in absorbance of RNO monitored at 350 nm for solutions containing [imidazole] = 8 x 10^{-3} M, [RNO] = 4 x 10^{-5} M and varying amounts of PABA irradiated for 15 minutes.

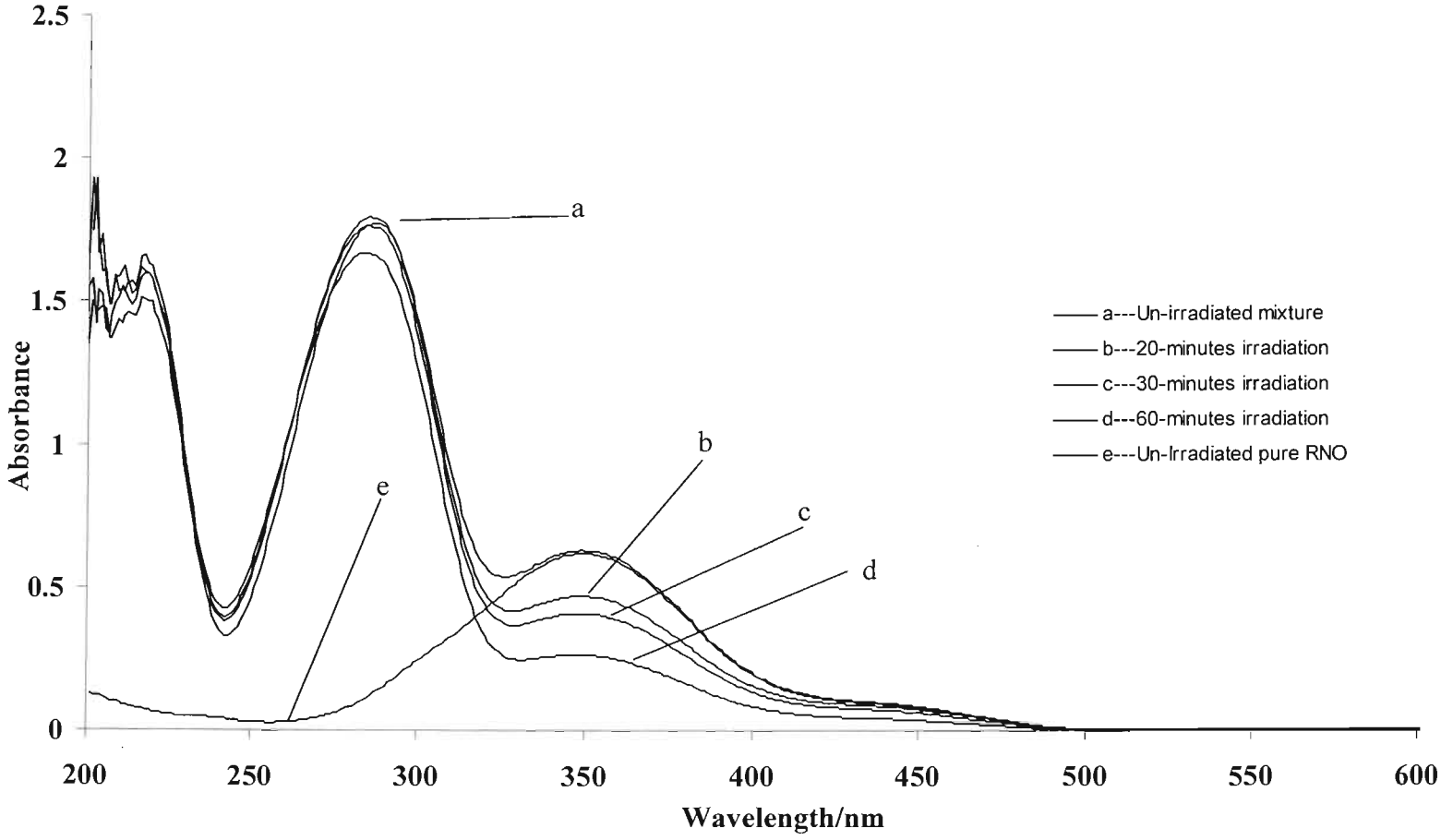

Figure 2.50: UV spectra of an un-irradiated solution of [RNO] = 4 x 10^{-5} M, an un-irradiated mixture of [PABA] = 1.5 x 10^{-4} M, [imidazole] = 1.6 x 10^{-3} M and [RNO] = 4 x 10^{-5} M and this mixture irradiated for varying time intervals. All spectra were obtained in a 10 mm pathlength quartz cuvette.

enough to completely bleach RNO. As explained earlier, consumption of more than 10 to 15% of RNO is not desirable **[248]**. A 15-minute irradiation period was found to be the optimal irradiation time. It gave a measurable change in absorbance and less than 15% of the original RNO was consumed. Irradiation times ranging from 2 minutes to a maximum of 20 minutes were used for those experiments in which irradiation time was varied. Table 2.20 shows the absorbance and change in absorbance of a solution containing only RNO and that of a solution containing RNO, PABA and imidazole that have been irradiated for the range of times selected. These values are presented in Figures 2.51 and 2.52.

Table 2.20: Absorbance at 350 nm for variable irradiation times of a solution of [RNO] = 4 x 10^{-5} M and a solution containing [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and [PABA] = 1 x 10^{-3} M at pH 3.0.

Irradiation Time/minutes	Absorbance		Change in Absorbance	
	RNO	Mixture	RNO	Mixture
0	0.6235	0.6721	0	0
2	0.5909	0.6057	0.0326	0.0664
4	0.5837	0.5541	0.0398	0.1180
6	0.5772	0.4982	0.0463	0.1739
10	0.5674	0.4233	0.0561	0.2488
14	0.5601	0.3573	0.0634	0.3148
17	0.5557	0.3067	0.0678	0.3654
20	0.5613	0.2622	0.0622	0.4099

From the spectra and plots shown in the previous paragraphs, we can conclude that imidazole and RNO could be used for quantification of PABA-photosensitised singlet oxygen generated in the presence of thymine.

Although the literature **[248]** indicates that an RNO concentration of 4 x 10^{-5} M is optimal we had to confirm whether it was indeed so for our investigation. Therefore, mixtures of imidazole of concentration 8 x 10^{-3} M and PABA of concentration 1 x 10^{-3} M with varying amounts of RNO were irradiated for fifteen minutes. Table 2.21 shows the absorbance and change in absorbance

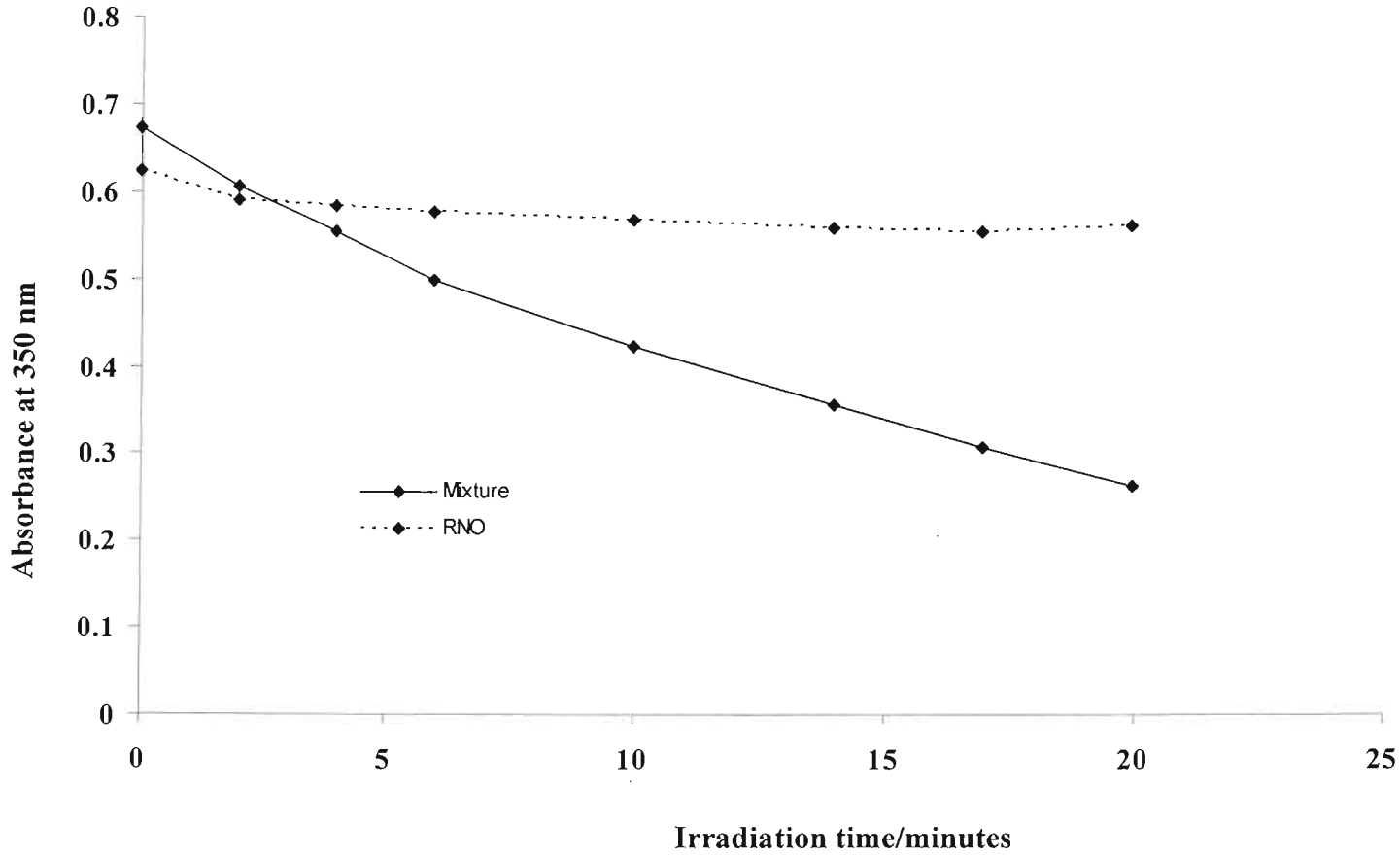

Figure 2.51: Absorbance of RNO monitored at 350 nm for a solution of [RNO] = 4 x 10^{-5} M and a solution containing [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and [PABA] = 1 x 10^{-3} M at pH 3.0 obtained in a 10 mm pathlength cuvette for different irradiation times.

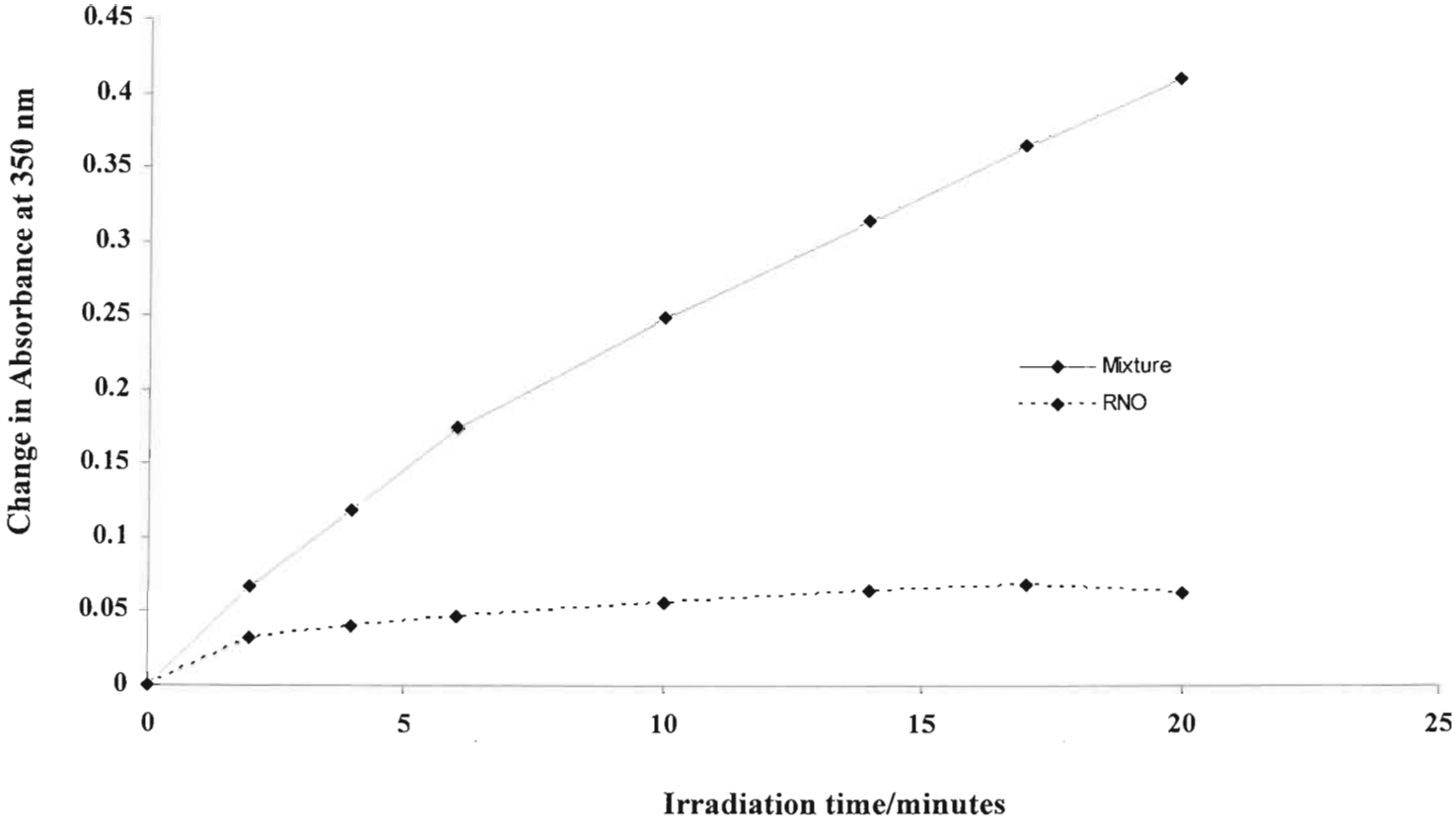

Figure 2.52: Change in absorbance of RNO monitored at 350 nm for a solution of [RNO] = 4 x 10^{-5} M and a solution containing [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and [PABA] = 1 x 10^{-3} M at pH 3.0 obtained in a 10 mm pathlength cuvette for different irradiation times.

values monitored at 350 nm for this set of experiments. The values are plotted in Figures 2.53 and 2.54.

Table 2.21: Absorbance measurements at 350 nm for 15-minutes irradiation of a solution of [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M and varying RNO concentrations at pH 3.0.

[RNO]/10^{-3} M	Absorbance	Change in Absorbance
5.00	0.1469	0.3584
4.00	0.1521	0.2052
3.00	0.1401	0.1060
2.50	0.1425	0.0908
1.25	0.1176	0.0322
1.00	0.1015	0.0185
0.50	0.0843	0.0135

As seen from the plot in Figure 2.53, the optimal value for the RNO concentration is about 4 x 10^{-5} M because after this concentration value, there is no increase in the absorbance, as would have been expected. This agrees with the literature value used by other researchers **[248, 250, 251, 252]**. Although RNO could be bleached by singlet oxygen at still higher concentrations as seen in Figure 2.54 but some literature reports indicate that side reactions are likely to take place for concentrations higher than 4 x 10^{-5} M **[251]**.

Since our investigation involved both vitamin C and vitamin E, we also had to perform a preliminary investigation to determine whether there was any interaction between them and RNO.

Absorbance measurements at 350 nm of un-irradiated solutions containing RNO, imidazole and a vitamin were recorded at different time intervals over a period of two hours. The reason for this was to investigate the stability of the components in the solution before irradiation. The concentrations used were 4 x 10^{-5} M, 8 x 10^{-3} M, 0.018 g L^{-1} and 0.044 g L^{-1} for RNO, imidazole, vitamin E

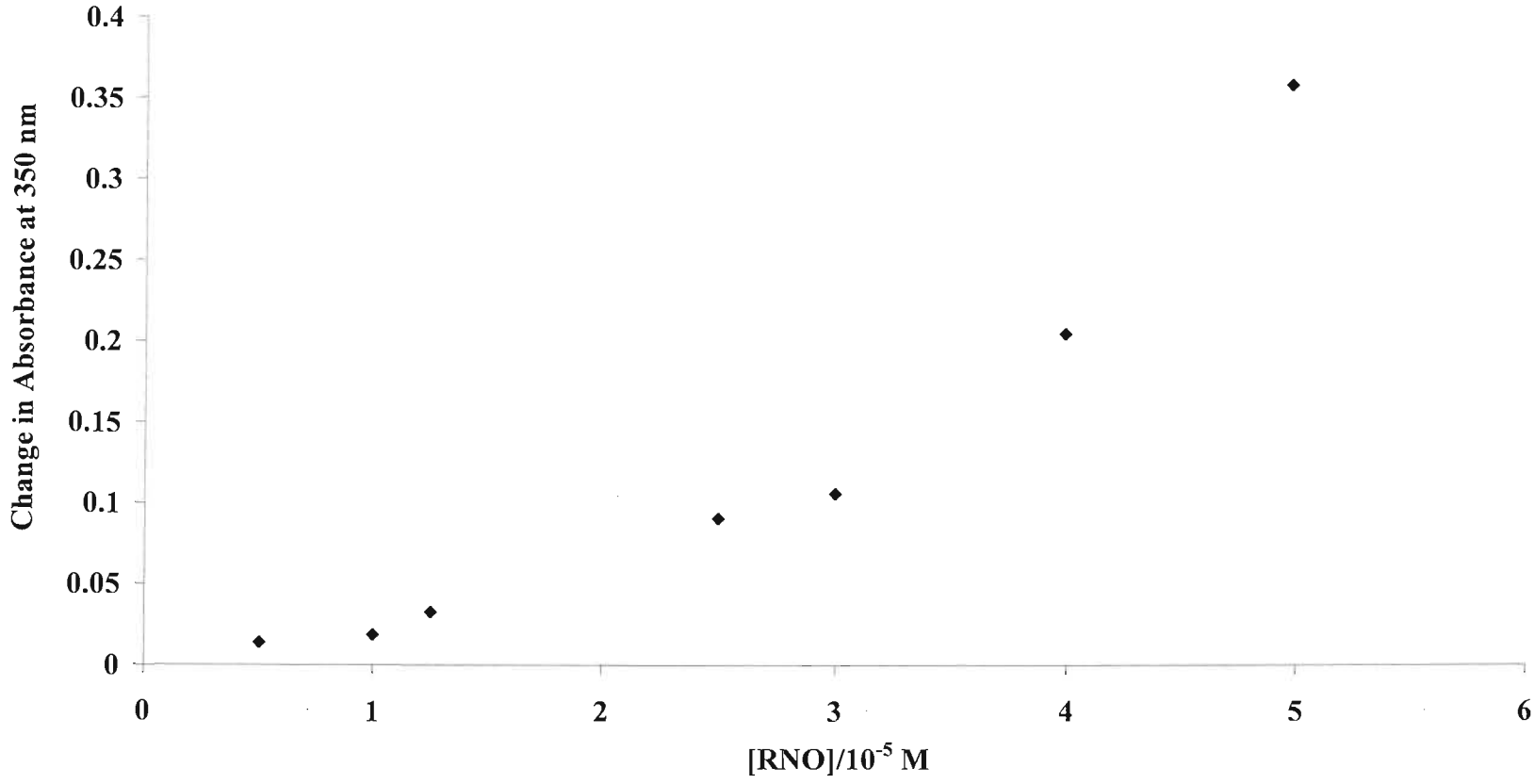

Figure 2.54: Change in absorbance at 350 nm for a mixture of [imidazole] = 8 x 10^{-3} M, [PABA] = 10^{-3} M and varying concentrations of RNO at pH 3.0 irradiated in a 10 mm pathlength quartz cuvette using the Osram HBO 500 W high pressure mercury lamp and pyrex filter combination.

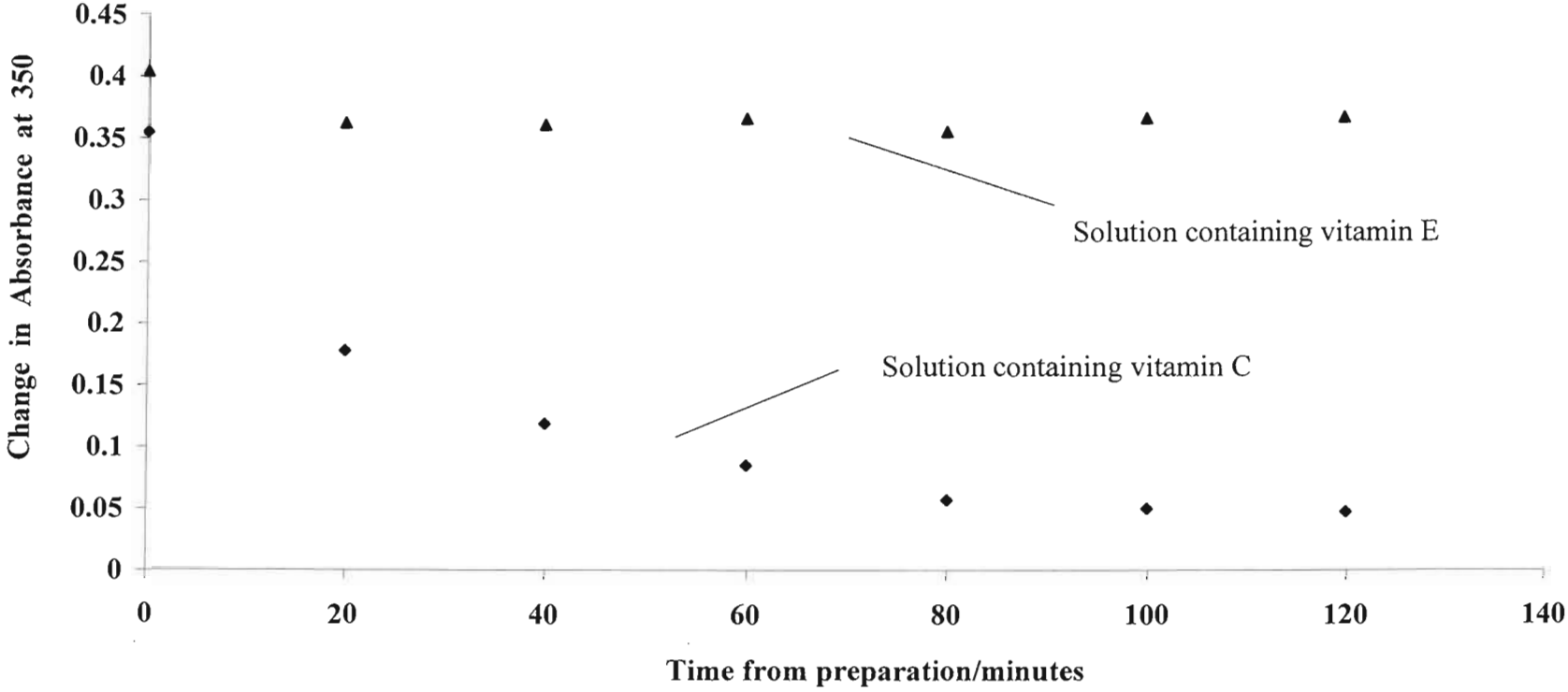

Figure 2.55: Change in absorbance at 350 nm for an un-irradiated solution containing [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and a vitamin at pH 3.0 contained in a 10 mm pathlength cuvette. The concentrations of the vitamins were [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

and vitamin C, respectively. Values of the absorbance of these solutions are shown in Table 2.22 and plotted in Figure 2.55.

Table 2.22: Absorbance measurements at 350 nm over a period of time for un-irradiated samples containing [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and a vitamin at pH 3.0 The concentrations of the vitamins were [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

Time from preparation/ minutes	Absorbance of vitamin C mixture	Absorbance of vitamin E mixture
0	0.3551	0.4044
20	0.1788	0.3631
40	0.1196	0.3622
60	0.0860	0.3674
80	0.0576	0.3565
100	0.0510	0.3678
120	0.0480	0.3685

From Figure 2.55, it is evident that vitamin E is stable in the presence of imidazole and RNO but vitamin C is not stable under the same conditions.

Figure 2.56 shows three spectra. One spectrum for an un-irradiated mixture of PABA, imidazole and RNO. The other two spectra are for the un-irradiated mixture with either vitamin E or vitamin C added. The spectrum that represents the presence of vitamin C is highest in absorbance followed by the spectrum representing the presence of vitamin E and lastly the spectrum for the mixture that contains no vitamins. The high absorbance in the two spectra is attributed to the additive absorption property of the mixtures. It shows that the mixture with vitamin C does not absorb much radiation above 300 nm. However, the absorbance of the solution containing vitamin E is highest.

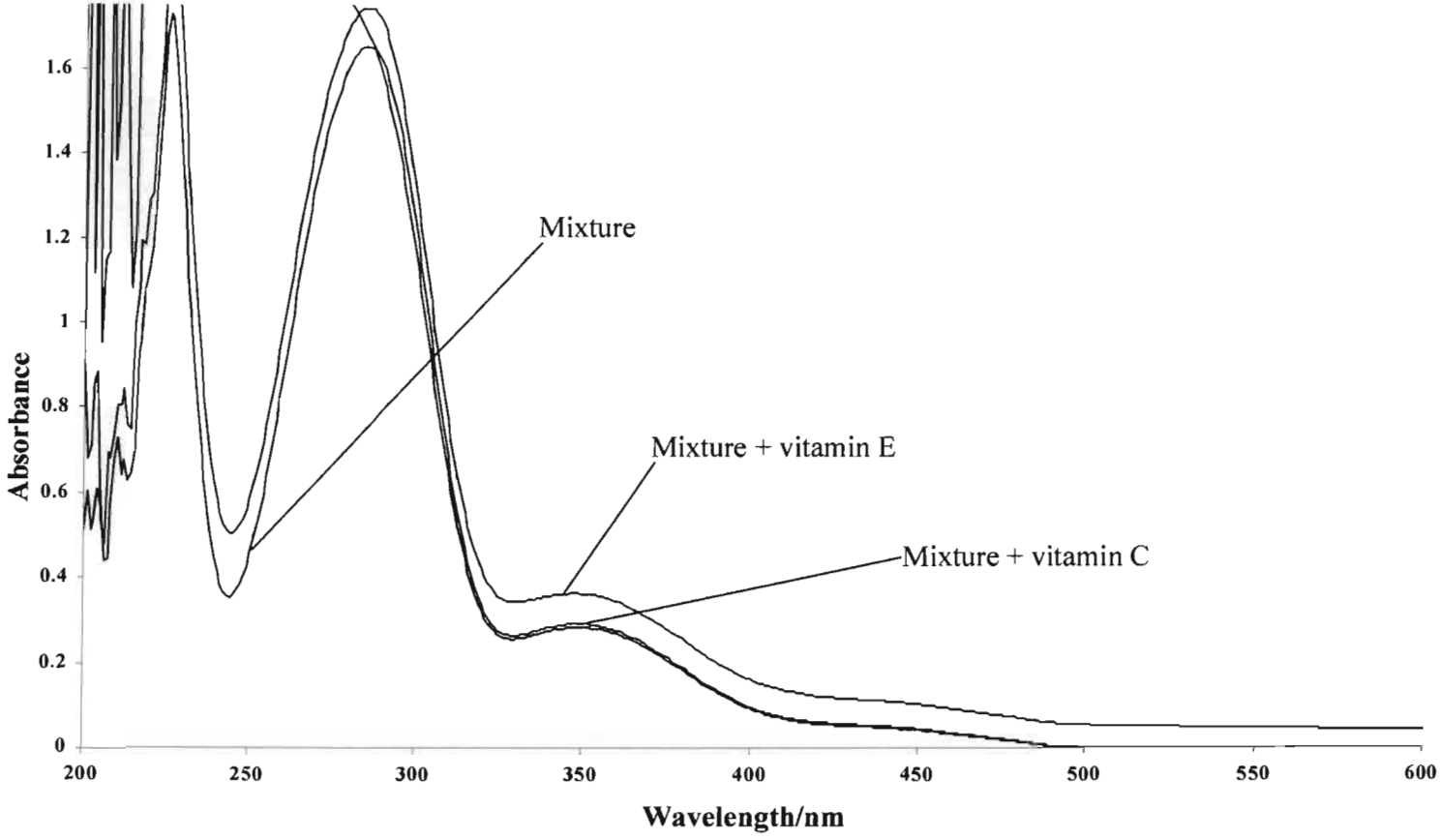

Figure 2.56: UV spectra for an un-irradiated aqueous solution containing [PABA] = 1.5 x 10^{-4} M, [imidazole] = 1.6 x 10^{-3} M and [RNO] = 4.0 x 10^{-5} M, and either [vitamin E] = 0.018 g L^{-1} or [vitamin C] = 0.044 g L^{-1} at pH 3.0 obtained in 10 mm pathlength quartz cuvette. The spectrum labeled "mixture" is for PABA, imidazole and RNO at the same concentration but without addition of vitamins.

Figure 2.57 shows spectra for an un-irradiated sample of RNO alone and un-irradiated samples containing RNO and vitamin E. The UV spectra of the samples were taken over a period of time. These two spectra are superimposed on one another. The other spectra are for an unirradiated sample containing RNO and vitamin C taken at different times from preparation. The spectrum for RNO solution was recorded immediately after preparation whereas the spectrum for the solution that contained vitamin E was taken after 35 minutes. From the two spectra, there is no noticeable change in the absorbance due the presence of vitamin E. This implies that for the time period considered, addition of vitamin E does not change the spectrum of RNO indicating that vitamin E does not react with RNO, at least for the first 35 minutes. This period is almost double the maximum irradiation period of 20 minutes that was used in our experiments. The spectra for RNO and vitamin C solution mixture show a gradual decrease in absorbance from the time of preparation of the solution over a period of 158 minutes. This indicates that vitamin C reacts with RNO over a period of time even without irradiation. To confirm that the bleaching is due to vitamin C and not imidazole, measurements for an un-irradiated mixture of RNO and vitamin C were taken over a period of time. The concentrations of all the species were the same as those used in the previous investigations. The absorbance values of RNO at 350 nm are shown in Table 2.23 and plotted in Figure 2.58. There is a linear decrease in absorbance against time confirming that vitamin C bleaches RNO even in the absence of UV radiation. This confirms that there is interaction between the two species. Therefore, all absorbance measurements that involved vitamin C had to be corrected for the change in absorbance for the un-irradiated sample.

Table 2.23: Absorbance measurements at 350 nm for an un-irradiated solution containing [RNO] = 4 x 10^{-5} M and [vitamin C] = 0.018 g L^{-1} at pH 3.0 recorded at various time interval after preparation of the solutions.

Time/minutes	Absorbance	Time/minutes	Absorbance
10	0.7725	80	0.6073
20	0.7470	100	0.5703
40	0.7014	120	0.5143
60	0.6518		

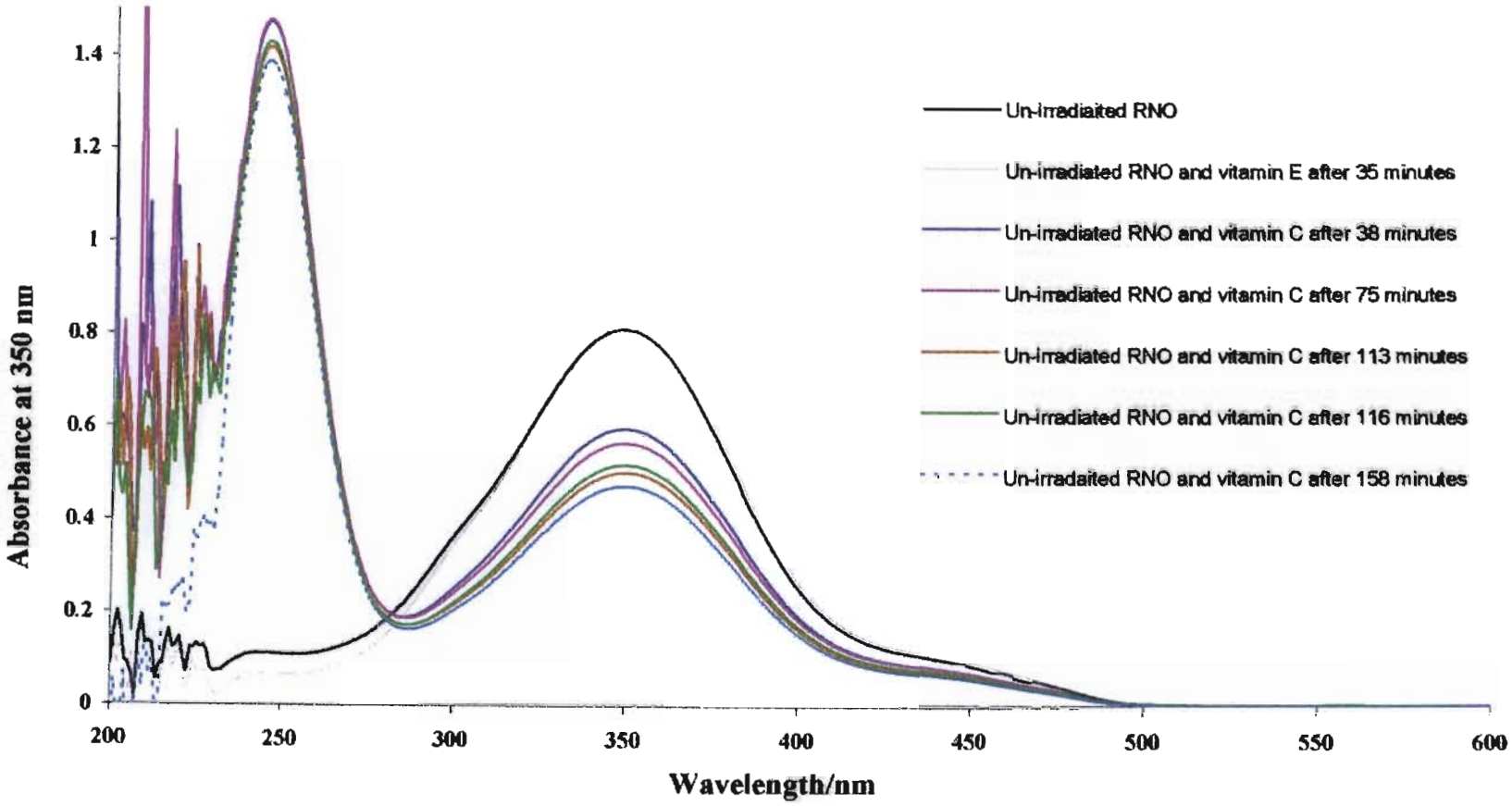

Figure 2.57: UV spectra of un-irradiated solutions containing [RNO] = 4.0×10^{-5} M, [RNO] = 4.0×10^{-5} M and [vitamin E] = 0.018 g L^{-1}, and [RNO] = 4.0×10^{-5} M and [vitamin C] = 0.044 g L^{-1} obtained in a 10 mm pathlength quartz cuvette.

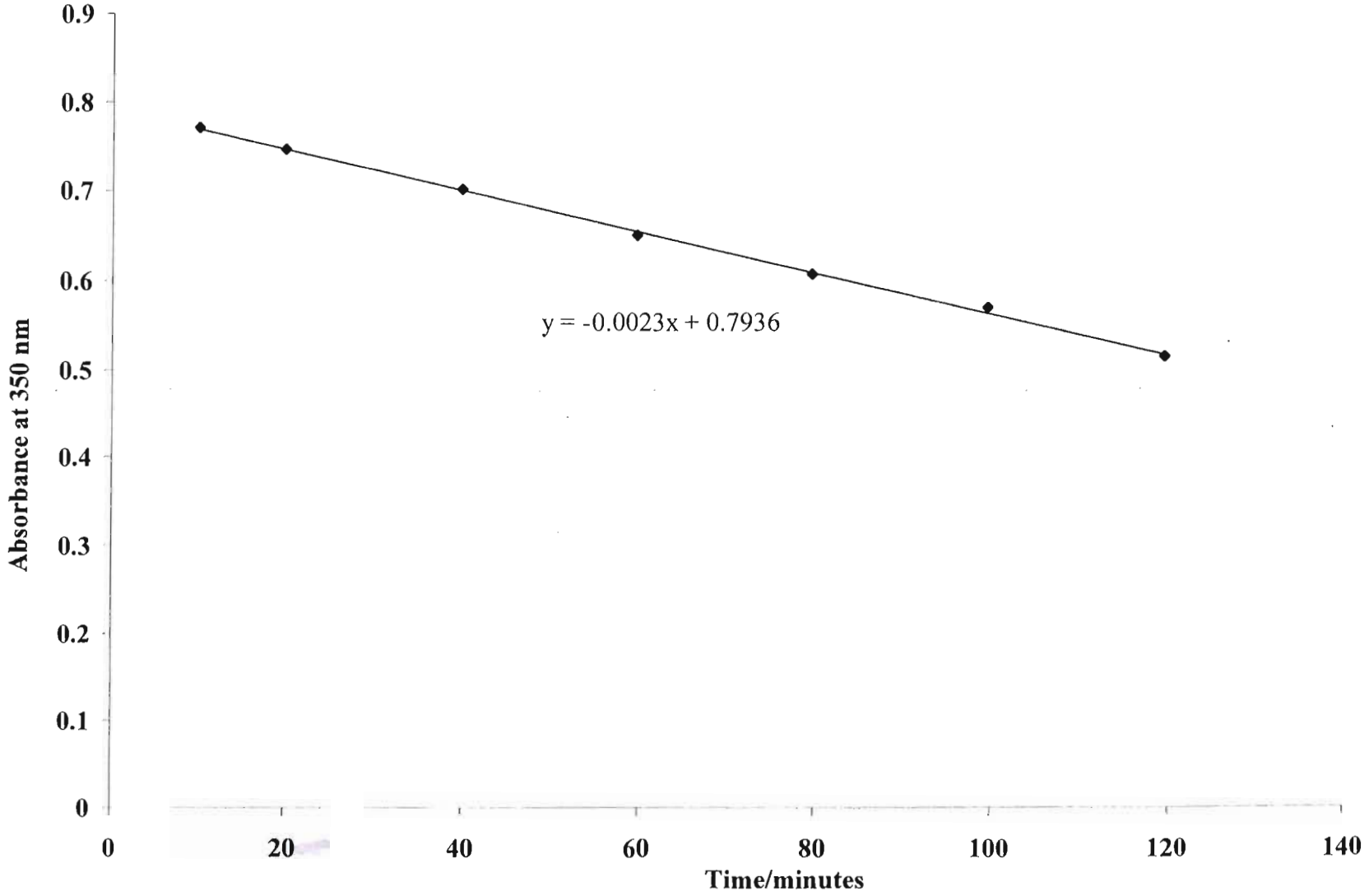

Figure 2.58: Absorbance at 350 nm of an un-irradiated solution containing [RNO] = 4 x 10^{-5} M and [vitamin C] = 0.044 g L^{-1} obtained in a 10 mm pathlength quartz cuvette.

After considering the results from the preceding paragraphs, we can conclude that RNO together with imidazole can be used to determine the effect of the two vitamins on PABA-photosensitised formation of singlet oxygen. Details of the experimental procedure are explained in the next sections.

2.9.3 EXPERIMENTAL PROCEDURE

Millipore water was used to prepare the buffer and sample solutions. A pH 3.0 phosphate buffer was prepared by mixing 0.20 M Na_2HPO_4 and 0.1 M citric acid in the ratio of 20.5 to 79.5 to give a final pH in the range of 3.0-3.1 and a concentration of 0.002 M for phosphate ion. Stock solutions of RNO, imidazole, PABA and thymine were prepared in the buffer every two weeks. Fresh stock solutions of vitamin C and vitamin E were prepared on each day of use.

To prepare stock solutions, required amounts of the reagents were weighed and dissolved in the phosphate buffer. Irradiation samples were prepared by serial dilution of the stock solution. The solutions used for singlet oxygen studies were not purged with nitrogen. This was to make sure that an air-saturated environment was present for singlet oxygen formation. The sample solutions were protected from light by covering them with aluminum foil. The mixtures prepared were placed in a quartz cell with an optical pathlength of 10 mm (Figure 2.5). The solutions were irradiated with the Osram HBO 500 W high pressure mercury lamp in combination with the 10 mm thick Pyrex filter (Figure 2.3). After irradiation, the cuvette containing the irradiated sample was shaken thoroughly to ensure a uniform solution. Absorbance measurements of these solutions were determined at 350 nm on the Perkin-Elmer Lambda 35 UV spectrophotometer before and after irradiation. The absorbance of the irradiated sample was subtracted from the absorbance of the un-irradiated sample to find the change in absorbance. The change in absorbance was plotted as a function of either concentration or irradiation time.

2.9.4 EXPERIMENTAL CONDITIONS

The following is a summary of the concentrations and irradiation times used in the singlet oxygen investigation. The following concentrations were constant: RNO, 4 x 10^{-5} M; PABA, 1 x 10^{-3} M; imidazole, 8 x 10^{-3} M; thymine, 1 x 10^{-2} M, vitamin E; 0.018 g L^{-1} and vitamin C, 0.044 g L^{-1}. The irradiation time was constant at 15 minutes. For variable parameters, irradiation time ranged between 2 minutes and 20 minutes while the concentration of PABA was varied from 1.5 x 10^{-2} M to 3.0 x 10^{-5} M. The concentration of thymine was varied from 1 x 10^{-3} to 10 x 10^{-3}M, while those for vitamins E and C were varied from 0.003 g L^{-1} to 0.030 g L^{-1} and 0.011 g L^{-1} to 0.108 g L^{-1}. The exact concentrations and irradiation times used are tabulated in Tables 2.24 to 2.30.

Table 2.24: Concentrations of a solution of PABA, vitamin E, vitamin C, [RNO] = 4 x 10^{-5} M and [imidazole] = 8 x 10^{-3} M at pH 3.0 irradiated for varying times in a 10 mm pathlength quartz cuvette with an Osram HBO 500 W high pressure mercury lamp in combination with the pyrex filter.

[PABA]/ M	[Vitamin E]/ g L^{-1}	[Vitamin C]/ g L^{-1}	Irradiation time/ minutes
1 x 10^{-3}	0	0	2, 4, 6, 10, 14, 17 and 20
1 x 10^{-3}	0.018	0	2, 4, 6, 10, 14, 17 and 20
1 x 10^{-3}	0	0.044	2, 4, 6, 10, 14, 17 and 20
1 x 10^{-3}	0.018	0.044	2, 4, 6, 10, 14, 17 and 20

Table 2.25: Concentrations of a solution of thymine, PABA, vitamin E, vitamin C, [RNO] = 4 x 10^{-5} M and [imidazole] = 8 x 10^{-3} M at pH 3.0 irradiated for varying times in a 10 mm pathlength quartz cuvette with an Osram HBO 500 W high pressure mercury lamp in combination with the pyrex filter.

[Thymine]/M	[PABA]/ M	[Vitamin E]/ g L^{-1}	[Vitamin C]/ g L^{-1}	Irradiation time/ minutes
1 x 10^{-2}	1 x 10^{-3}	0	0	2, 4, 6, 10, 14, 17 and 20
1 x 10^{-2}	1 x 10^{-3}	0.018	0	2, 4, 6, 10, 14, 17 and 20
1 x 10^{-2}	1 x 10^{-3}	0	0.044	2, 4, 6, 10, 14, 17 and 20
1 x 10^{-2}	1 x 10^{-3}	0.018	0.044	2, 4, 6, 10, 14, 17 and 20

Table 2.26: Concentrations of a mixture of PABA, vitamin E, vitamin C, [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and varying PABA concentrations at pH 3.0 irradiated for 15 minutes in a 10 mm pathlength quartz cuvette with an Osram HBO 500 W high pressure mercury lamp in combination with the Pyrex filter.

[PABA]/10^{-2} M	[Vitamin E]/g L^{-1}	[Vitamin C]/g L^{-1}
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	0	0
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	0.018	0
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	0	0.044
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	0.018	0.044

Table 2.27: Concentrations of a mixture of thymine, PABA, vitamin E, vitamin C, [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and varying PABA concentrations at pH 3.0 irradiated for 15 minutes in a 10 mm pathlength quartz cuvette with an Osram HBO 500 W high pressure mercury lamp in combination with the Pyrex filter.

[PABA] /10^{-2} M	[Thymine]/ M	[Vitamin E] /g L^{-1}	[Vitamin C] /g L^{-1}
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	1 x 10^{-2}	0	0
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	1 x 10^{-2}	0.018	0
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	1 x 10^{-2}	0	0.044
1.5, 1.2, 0.9, 0.6, 0.3, 0.15, 0.003	1 x 10^{-2}	0.018	0.044

Table 2.28: Concentrations of a mixture of PABA, vitamin E, vitamin C, [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M and varying thymine concentrations at pH 3.0 irradiated for 15 minutes in a 10 mm pathlength quartz cuvette with an Osram HBO 500 W high pressure mercury lamp in combination with the Pyrex filter.

[Thymine]/10^{-3} M	[PABA]/ M	[Vitamin E]/ g L^{-1}	[Vitamin C]/ g L^{-1}
1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1 x 10^{-3}	0	0
1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1 x 10^{-3}	0.018	0
1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1 x 10^{-3}	0	0.044
1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1 x 10^{-3}	0.018	0.044

Table 2.29: Concentrations of vitamin E varied in a mixture of [PABA] = 1 x 10^{-3} M, [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M at pH 3.0 in the absence and presence of vitamin C and thymine irradiated for 15 minutes in a 10 mm pathlength quartz cuvette with an Osram HBO 500 W high pressure mercury lamp in combination with the Pyrex filter. The concentrations were [thymine] = 1 x 10^{-2} M and [vitamin C] = 0.044 g L^{-1}.

[Vitamin E]/g L^{-1}	[Vitamin E]/g L^{-1}
0.003	0.018
0.006	0.021
0.009	0.024
0.012	0.030
0.015	

Table 2.30: Solution of vitamin C of [PABA] = 1 x 10^{-3} M, [RNO] = 4 x 10^{-5} M, and [imidazole] = 8 x 10^{-3} M and variable vitamin C concentration at pH 3.0 in the absence and presence of both vitamin C and thymine irradiated for 15 minutes in a 10 mm pathlength quartz cuvette with an Osram HBO 500 W high pressure mercury lamp in combination with the Pyrex filter combination. The concentrations were [thymine] = 1 x 10^{-2} M and [vitamin E] = 0.018 g L^{-1}.

[Vitamin C]/g L^{-1}	[Vitamin C]/g L^{-1}
0.010	0.066
0.022	0.076
0.032	0.088
0.044	0.0.98
0.054	0.108

The results for these experiments are presented in Appendix D and discussed in Section 3.2.

2.10 EXPERIMENTAL DIFFICULTIES

Some experimental difficulties were encountered during the investigation of the PABA-photosensitised dimerisation of thymine in aqueous media in the presence of the two antioxidants. Although the Vitamin E acetate used was water soluble, the solubility was very limited hence the effect of varying the concentration of Vitamin E could not be studied effectively since the range covered was small. Vitamin C is susceptible to destruction by UV radiation. Studies on the effect of vitamin C at long irradiation times such as 24 hours cannot be accurate since a very small amount of the original Vitamin C remains in the system. At very long irradiation times, vitamin C is consumed to such an extent that its concentration is below the limit of detection. To determine the maximum effect of a particular reagent required long irradiation times such as 24 hours. In the case of vitamin C, it was not possible to accurately infer its effect at long irradiation times.

PABA is a poor photosensitiser relative to others. It absorbs poorly at wavelengths above 325 nm with a low quantum yield of 0.07 for intersystem crossing from its singlet to its triplet state. Therefore to obtain large yields requires very long irradiation times. But very long irradiation times imply that more photoproducts from PABA are formed. To reach a maximum dimer yield would require 70-80 hours **[74]**. This was not feasible given the short lifetime of 400 effective hours for the Osram HBO 500 W high pressure mercury lamp. The poor sensitisation and formation of adducts resulted in very low dimer yields that were close to the detection limit of the PDA.

At low pH, 2% of the thymine dimer solution dissociates back to thymine monomer **[257]**. This fact further reduces the amount of dimer yield that could be detected in the investigation. The low dimer yields gave very small peaks, which in turn resulted in large errors in the peak-integration process. With detection of dimers at concentration as low as 1×10^{-6} M, baseline stability was a problem. Baseline stability affects the accuracy especially where the yield is decreasing. These large errors are evident from the large error bars plotted in the graphs.

The photolysis cell had a volume of 480 µl. When the contents were transferred into the vials of the HPLC autosampler and two 80 µl injections made, the level went below the lowest level the syringe could reach. Hence there was insufficient sample to test precision and during optimisation more time had to be spent on irradiating more solutions. This problem was not encountered with U6K manual injector.

CHAPTER 3

RESULTS AND DISCUSSION

The work in this thesis aims at investigating the PABA-photosensitised dimerisation of thymine and the effects of the presence of vitamin E and vitamin C in aqueous solution. It also aims at investigating the interaction of vitamin E and vitamin C with singlet oxygen generated by PABA in aqueous solutions. The preceding chapter detailed the experimental techniques used to gather the necessary data. All samples were maintained at pH 3.0 and purged with nitrogen to remove dissolved oxygen except for the experiments involving measurement of singlet oxygen. Irradiations were carried out at wavelengths greater than 300 nm to simulate the solar energy that is incident on the earth's surface and ensure that dimerisation and singlet oxygen formation occurred via the photosensitisation route. This chapter presents and discusses the results obtained. It falls into two main parts. The first part discusses the photosensitised dimerisation of thymine and the second part discusses the photosensitised formation of singlet oxygen. In both photosensitised processes, comparisons are made between the effect of the presence in solution of the two antioxidants and the absence of them.

3.1 PABA-PHOTOSENSITISED FORMATION OF THYMINE DIMERS

The following sections discuss the data obtained on thymine photodimerisation. The data obtained for these experiments are presented in Appendix C. Total thymine dimer yields were obtained as described in Section 2.7. The chromatograms and the associated UV-absorption spectra of the photoproducts for different sets of experiments are presented in the following sections.

3.1.1 EFFECT OF IRRADIATION TIME

The following section discusses the data on thymine dimer yield obtained when irradiation times were varied. Figure 3.1a shows dimer yield as a function of irradiation time for constant initial concentrations of thymine, 1 x 10^{-2} M; PABA, 1 x 10^{-3} M; vitamin E, 0.018 g L^{-1} and vitamin C, 0.044 g L^{-1} at pH 3.0. Diagrams A, B, C and D in Figure 3.1b show the effect of absence of the vitamins, the presence of vitamins E, C and both vitamins respectively. Figures 3.2 to 3.7 show typical chromatograms and the associated UV-absorption spectra for photoproducts obtained from irradiation of these sets of samples.

Irradiation of thymine and PABA alone shows an increase in dimer yield with increase in irradiation time. The increase is approximately linear up to 9 hours. For irradiation times greater than 9 hours, the increase in yield is not linear but exponential. The chromatograms shown in Section 2.8 indicate large peak areas for un-reacted thymine and un-reacted PABA. This implies that there were no limiting reagents in the photochemical reaction between thymine and PABA. Prolonging the irradiation time increases the concentration of triplet PABA which in turn increases the concentration of triplet thymine. Both triplet thymine and triplet PABA are necessary for dimer yield. Triplet PABA is formed with a rate constant of 1.60 x 10^{9} M^{-1} s^{-1} **[65]**. Triplet thymine formation by direct absorption of light has a rate constant of 3.0 x 10^{7} M^{-1} s^{-1} **[46]**. This suggests that an increase in the concentration of triplet PABA by prolonging irradiation time increases the concentration of triplet thymine and consequently dimer yield. The combined increase in the triplet thymine and triplet PABA appears to overcome the effect of self-quenching of PABA, which reduces thymine yield and which is at its best at pH 3.0. For irradiation times greater than 9 hours, the linearity in the dimer yield is not observed. Instead, there is an exponential increase in the formation of the dimers. This is contrary to what Aliwell *et al.* **[258]** found. They found that the yield increases linearly for the whole range that they studied which was up to about 19 hours. The difference in their results and the results of our experiments can be attributed to the difference in the filters used. They used a 324 nm narrow band filter whereas we used a filter that allowed light of wavelength greater than 300 nm.

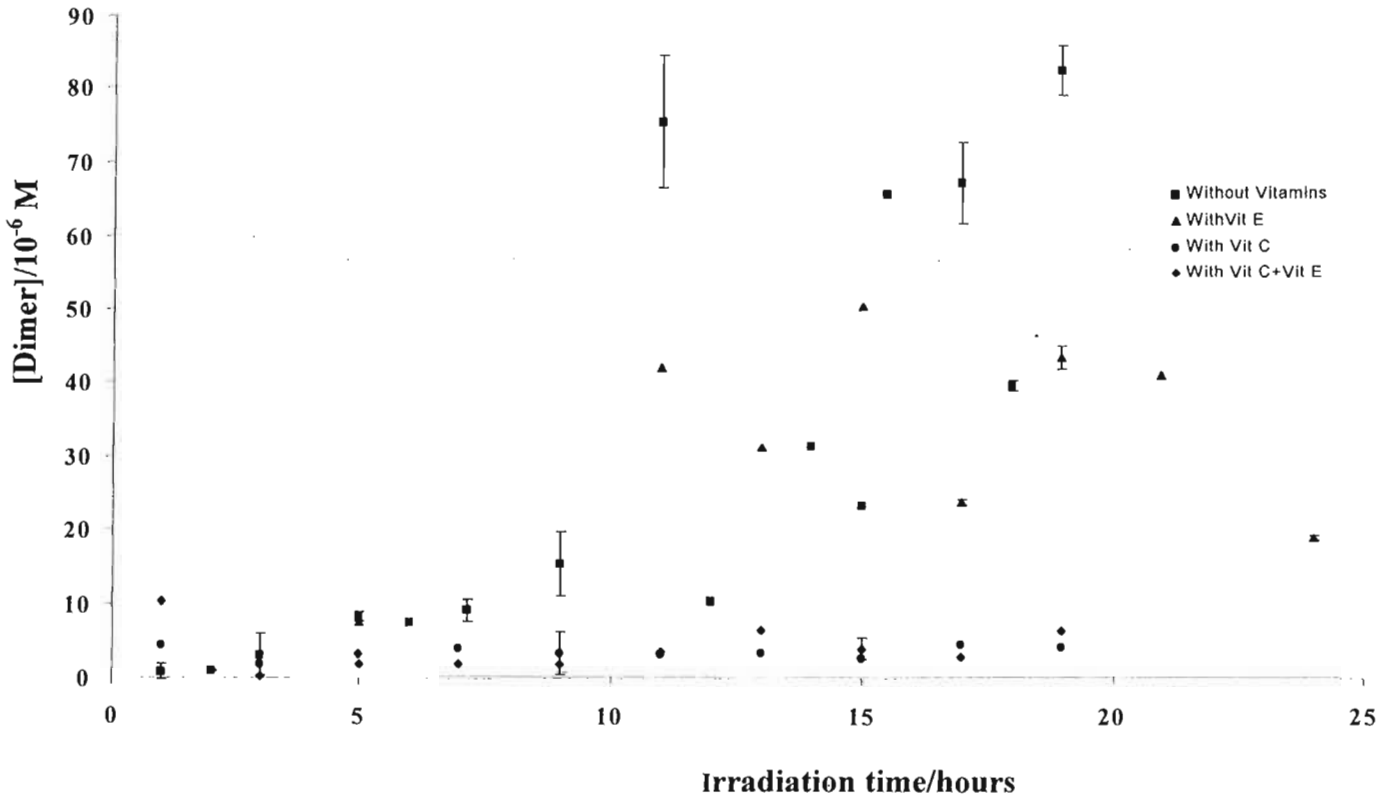

Figure 3.1a: Thymine dimer formation as a function of irradiation time, [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g l^{-1} and [vitamin C] = 0.044 g l^{-1} at pH 3.0. The error bars depict standard deviations for three replicate injections.

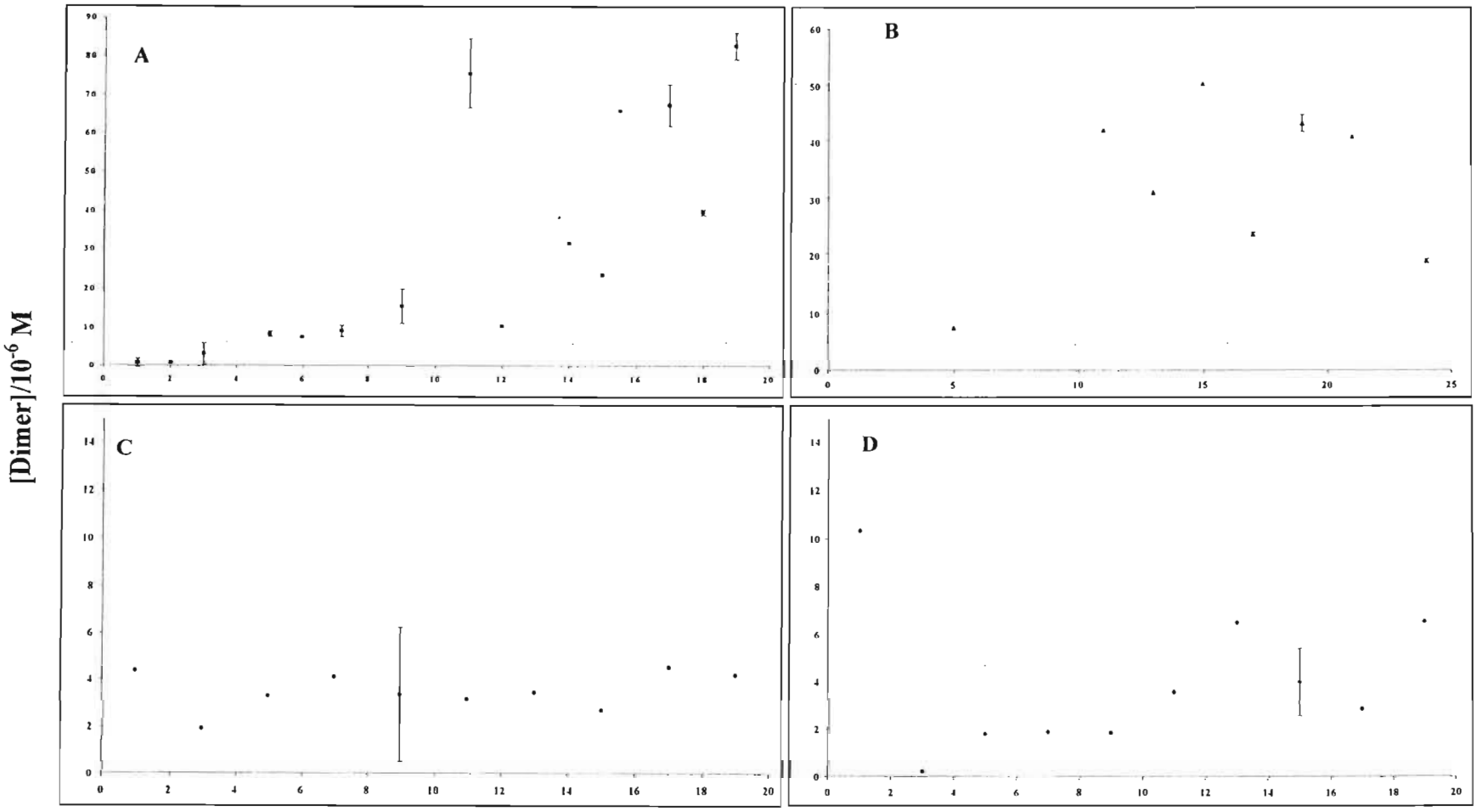

Figure 3.1b: **A**, no vitamins added; **B**, vitamin E added; **C**, vitamin C added; **D**, both vitamins E and C added.

Examples of the typical chromatograms and the associated UV-spectra obtained for this set of irradiations are shown in Figures 3.2 to 3.4

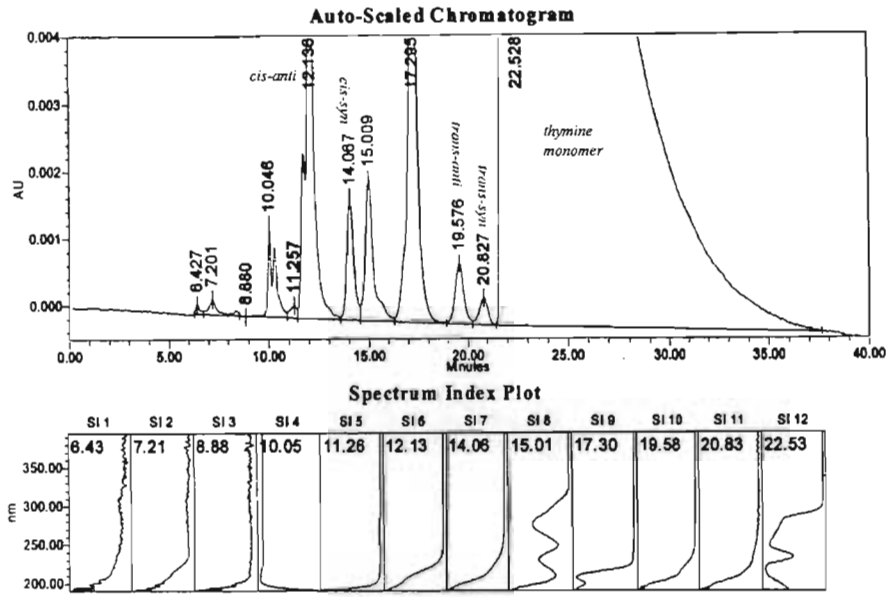

Figure 3.2: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M and [PABA] = 1 x 10^{-3} M at pH 3.0 irradiated for 15 hours and separated with an Ultracarb 5 ODS (20) column by using a 10% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

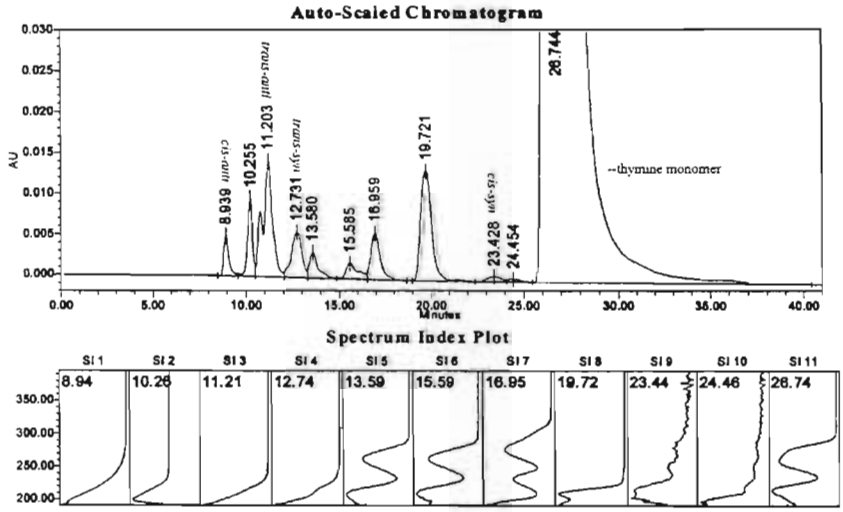

Figure 3.3: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M and [PABA] = 1 x 10^{-3} M at pH 3.0 irradiated for 15 hours and separated with an Ultracarb 5 ODS (20) column by using a 7% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

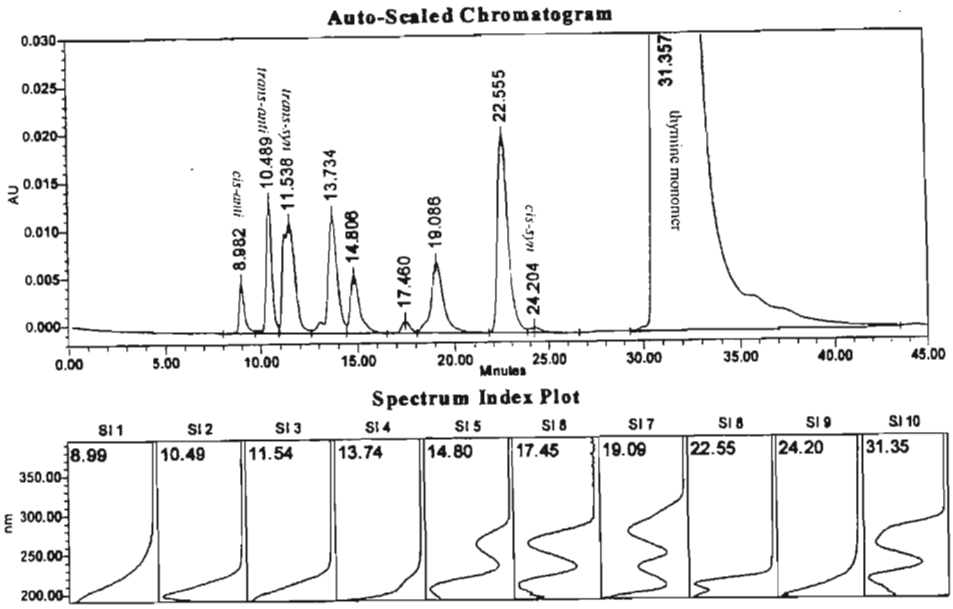

Figure 3.4: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M and [PABA] = 1 x 10^{-3} M at pH 3.0 irradiated for 17 hours and separated with an Ultracarb 5 ODS (20) column by using a 5% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

Introduction of vitamin E in the thymine and PABA system increases the dimer yield for irradiation times between 5 hours and 15 hours. For irradiation times longer than 15 hours vitamin E reduces the dimer yield. It is known that vitamin E shows positive activity, i.e. antioxidant activity, up to an optimal concentration above which, stability is lost and prooxidant effect comes into play **[191, 259, 260]**. The prooxidant activity can also be attributed to its susceptibility to undergo autoxidation and side reactions due to long hours of irradiation. At very high concentrations, side reactions may predominate and the antioxidant activity may be completely lost **[191, 261, 262]**. In Chapter 1 it was argued that vitamin E could act as a photosensitiser and that the mechanism by which vitamin E inhibits thymine dimer formation is not known. From our point of view, we suppose that it is highly likely that it could take a similar trend regarding concentration and photosensitising properties. Between 5 and 15 hours, the concentration of vitamin E is high and hence its prooxidant activity and photosensitising properties are prevalent resulting in an increase in dimer yield. At irradiation times longer than 15 hours, autoxidation and side

reactions could have lowered the concentration of vitamin E to an optimal value that resulted in lower dimer yield. This lower dimer yield can possibly be explained as follows. Vitamin E physically quenches or reacts with the triplet states of both thymine and PABA. It is quite possible that the presence of the vitamin also reduces the lifetime of these triplet states. These two mechanisms would terminate the reaction that would lead to thymine dimer formation. It is very unlikely that there would be a reduction in the rate of diffusion-controlled energy transfer as a result of increased viscosity of the system due to the presence of the vitamin. A typical chromatogram and the associated UV-spectra for this set of irradiation experiments are shown in Figure 3.5. The chromatogram shows no peak at about 12.76 minutes corresponding to vitamin E. This implies that vitamin E underwent self-oxidation or was destroyed by UV radiation to give photoproducts **[144, 145]**.

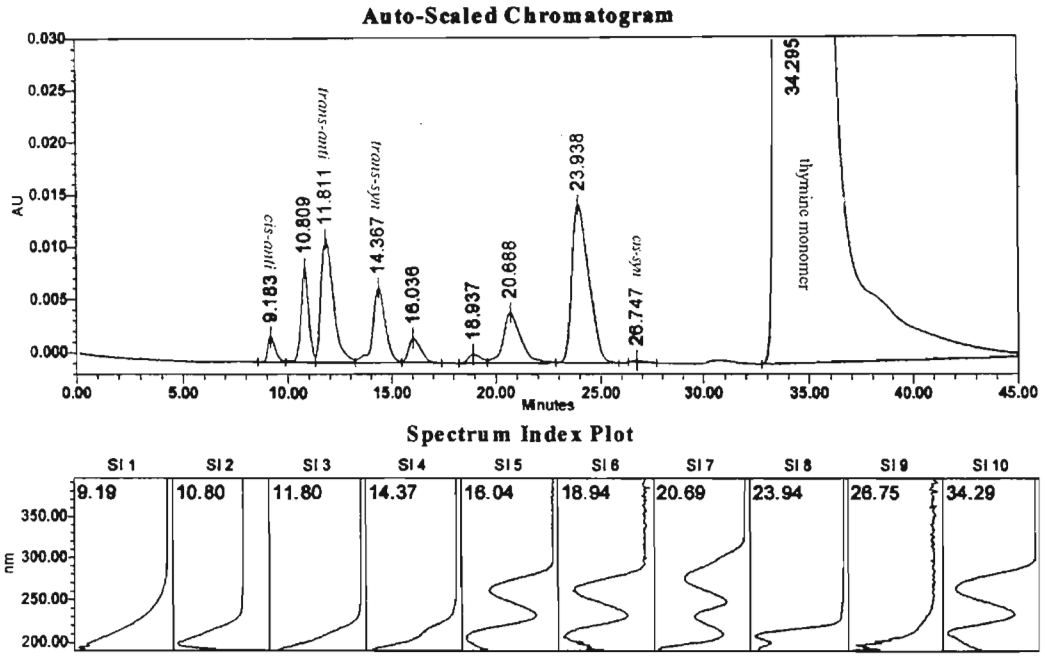

Figure 3.5: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin E] = 0.018 g L^{-1} at pH 3.0 irradiated for 21 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

Addition of vitamin C significantly lowers dimer yield. It lowers the yield throughout the range studied apart from the 1-hour irradiation. There are no reports in the literature showing the effects of vitamin C alone on thymine dimer formation. It is not clear how vitamin C alone reduces thymine dimer formation. We can only hypothesize that it is able to lower photosensitised thymine dimer formation because of how it interacts with the photosensitiser and triplet thymine. From Equation 3.1, vitamin C is able to intercept the triplet sensitiser and have the triplet photosensitiser dispense its energy **[177]**. It is possible that it can quench, react with triplet thymine or reduce its lifetime and thus reduce thymine dimer yield.

$$^3P^* + AsH^- + {}^3O_2 \rightarrow AscH^- + P + O_2^- + H^+ \qquad 3.1$$

Since a triplet photosensitiser is necessary for photosensitised dimerisation it would be logical to assume that once its population has been lowered, the dimer yield is also lowered. A typical chromatogram and the associated UV-spectra for this set of experiments are shown in Figure 3.6.

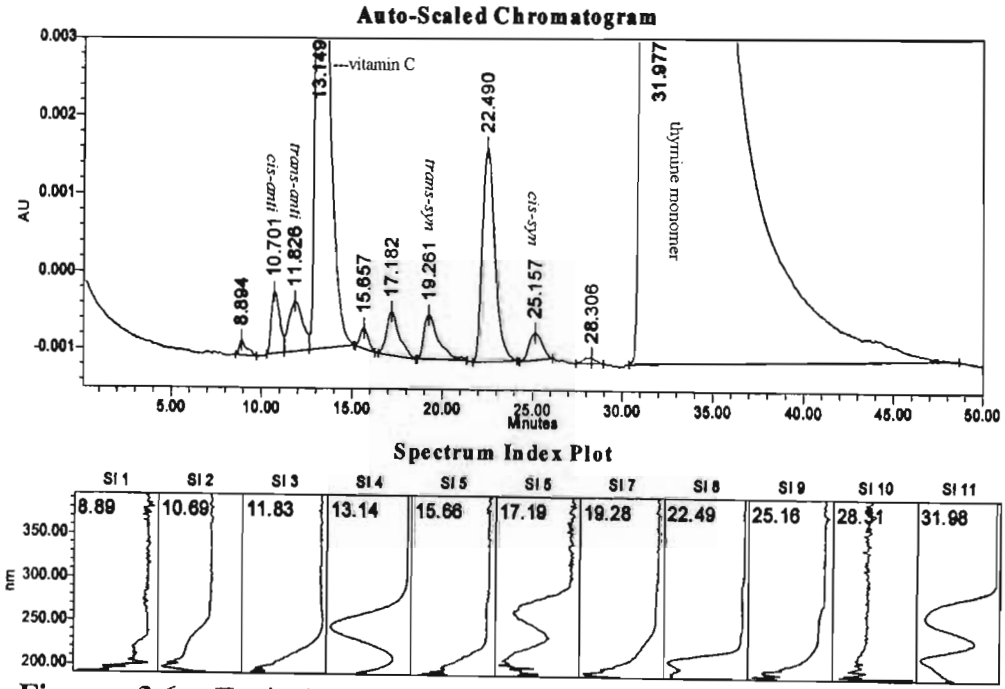

Figure 3.6: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin C] = 0.044 g L^{-1} at pH 3.0 irradiated for 19 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

The peak at 13.149 minutes is that of vitamin C. Since there was no vitamin E to regenerate ascorbic acid from ascorbate ions, the most likely reason is that the peak is for un-reacted vitamin C. This implies that the amount present was more than what was required to lower dimer yield and since it does not absorb UV radiation under the irradiation condition used it was not destroyed by that route.

Figure 3.7 shows a typical chromatogram and the associated UV-spectra for the irradiation of thymine, PABA, and vitamins E and C. Introducing vitamin E and vitamin C together has a significant effect on the dimer yield. It lowered the dimer yield throughout the range studied apart from the 1-hour irradiation. Studies on the combined oral intake of vitamins E and C **[170]** and their combined topical application **[86]** have shown similar trends regarding thymine dimer yield. This can be attributed to the synergism between vitamin C and vitamin E. Vitamin C is able to regenerate vitamin E that has been depleted by UV radiation. (Section 1.12.1 explains the regeneration.) Generally, the total dimer yield for the combined presence of the two vitamins is slightly lower than the case for the presence of vitamin C alone. Once again, the postulated mechanism that the vitamins can quench, react with triplets states of both thymine and PABA or reduce their lifetimes is most likely.

Discrepancies in the 1-hour irradiations can be attributed to low population of the available triplet photosensitiser. This results in very low dimer yields and gives large error during integration of the dimer peaks. The chromatogram shows the presence of neither vitamin E nor vitamin C. Their absence in the chromatograms suggests that they must have been used synergistically in lowering dimer yield. We would have expected a peak for un-reacted vitamin C but none is seen. This suggests that the excess vitamin C that would have given the peak was used to regenerate vitamin E **[177, 196, 197]**.

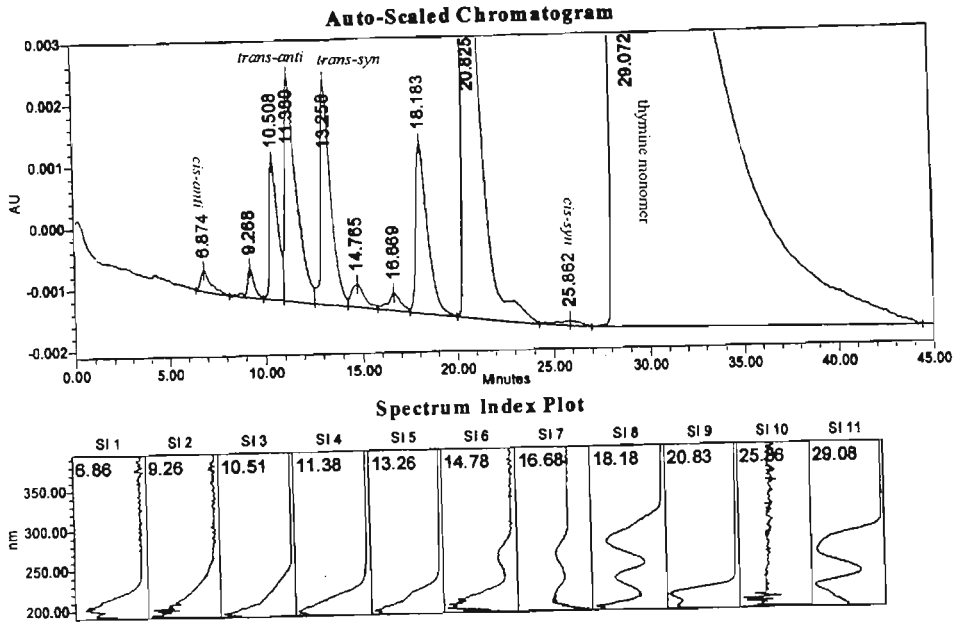

Figure 3.7: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0 irradiated for 9 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

Baseline resolution of peaks was achieved between individual dimers and also between dimers and other photoproducts. This is evident from the R values for resolution as summarised in Table 2.3. Figure 3.2 shows the chromatogram for photoproducts from irradiation of thymine and PABA whose chromatographic conditions were 1.5 mL min^{-1} and 10% (v/v) methanol. The order of elution of the dimer stereoisomers is consistent with that given by Cadet *et al.* **[213]** i.e. *cis-anti, cis-syn, trans-anti* and *trans-syn* and lastly thymine monomer. The different irradiation times, presence of the antioxidants and variation in concentrations gave different photoproducts on irradiation. Many other different photoproducts also eluted besides the dimer and thymine monomer. Separation also had to be achieved between any photoproduct and a dimer. Resolving a photoproduct and *trans-anti* thymine dimer presented a problem in most cases. The chromatographic conditions for the separation of thymine and PABA photoproducts shown in Figure 3.3 could not adequately resolve the peak for *trans-anti* dimer from a photoproduct properly (R = 0.9305).

Therefore other optimum conditions as seen in the caption of the figures had to be used in such cases. The flow rate was not altered but the methanol content of the eluent changed. Complete resolution of the peaks was necessary to minimise errors in peak integration, since the concentrations were very low. For this set of chromatograms, the R value for dimer peaks was greater than 1.5. These new conditions eluted the dimer in an order that was not consistent with the one given by Cadet *et al* **[213]**. The new order was *cis-anti, trans-anti, trans-syn, cis-syn* and lastly thymine monomer. This order had to be confirmed by separating standards of *trans-anti* and *cis-syn* thymine dimers. Since we did not have standards for the other two dimers, the order was always assumed as *cis-anti* followed by *trans-syn*. The peaks at 10.046 and 12.136 minutes in Figure 3.2 and 11.203 minutes in Figure 3.3 are split peaks. The software program allows peaks to be manually integrated by changing where a peak starts and where it ends in terms of time. Of course this happens if the UV-spectra of the split peaks are the same and in this case the width of the peak covers the two split peaks and therefore no area is left un-integrated.

In this set of experiments, the percentage yield of *cis-syn* dimer is lowest whereas that of *trans-syn* dimer is highest. Exceptions to this trend are seen in Figure 3.2 and Figure 3.6 where *trans-syn* dimer has the lowest percentage yield of the dimers. Generally, apart from some few exceptions, the yield is in the order *cis-syn* < *cis-anti* < *trans-anti* < *trans-syn*. Although there is a general pattern in the relative dimer yield, the presence of the vitamins do not appear to affect this pattern. In this set of experiments, all the parameters are the same except irradiation time which has been shown not to affect relative dimer yield **[34, 37, 38]**. In this set of chromatograms, other than the peaks for dimers, thymine and vitamins, any other peaks would represent photoproducts of PABA and the vitamins.

In conclusion, the dimer yield increases as the irradiation time increases. Individually vitamin C decreases thymine dimer yield whereas vitamin E does not. The presence of the two together lowers dimer yield significantly. To a great extent these results agree with what Placzek *et al.* **[169]** found regarding the combined effect of the two vitamins. They found that when taken orally

together, they lower the formation of thymine dimer. Lin *et al.* **[86]** also found similar results. They found that topical application of a combination of the two antioxidants reduces thymine dimer yield. Individual application did not produce the same effect. The presence of the vitamins does not alter the pattern of the relative yields of the stereoisomers of the dimer.

3.1.2 EFFECT OF PABA CONCENTRATION

The following section discusses the data on thymine dimer yield obtained when the concentration of PABA was varied. Figure 3.8a shows dimer yield as a function of initial PABA concentration for initial constant concentrations of thymine of 1 x 10^{-2} M, vitamin E, 0.018 g L^{-1} and vitamin C, 0.044 g L^{-1} at pH 3.0 and 6 hours of irradiation. Diagrams A, B, C and D in Figure 3.8b show the effect of absence of the vitamins, the presence of vitamins E, C and both vitamins respectively. Figures 3.9 to 3.12 show typical chromatograms and the associated UV-absorption spectra for photoproducts obtained for this set of experiments.

Irradiation of thymine and PABA alone shows a general decrease in dimer yield with increase in PABA concentration. Dimer yield for initial low concentration of PABA is higher than the yield at high initial PABA concentration. This trend in dimer yield can be explained in terms of an increase in efficiency of the self-quenching effect of triplet PABA in the unionised form at pH 3 **[65]**. An increase in PABA concentration increases self-quenching thus decreasing the amount of triplet PABA. Triplet PABA is necessary to populate triplet thymine, the precursor for dimerisation. Therefore, a decrease in triplet PABA implies that the dimer yield will also decrease. Another possible reason is the rate of photo absorption by PABA at different concentrations. At low PABA concentration, the rate of photon absorption is high and hence the dimer yield and vice versa for high concentration. For the concentration of thymine used, we would have expected base stacking to be more significant than self-quenching of triplet PABA and hence give a high dimer yield as suggested by the large rate constants for the photodimerisation of the thymine. Apparently this is not the case, i.e. the yield is low. An

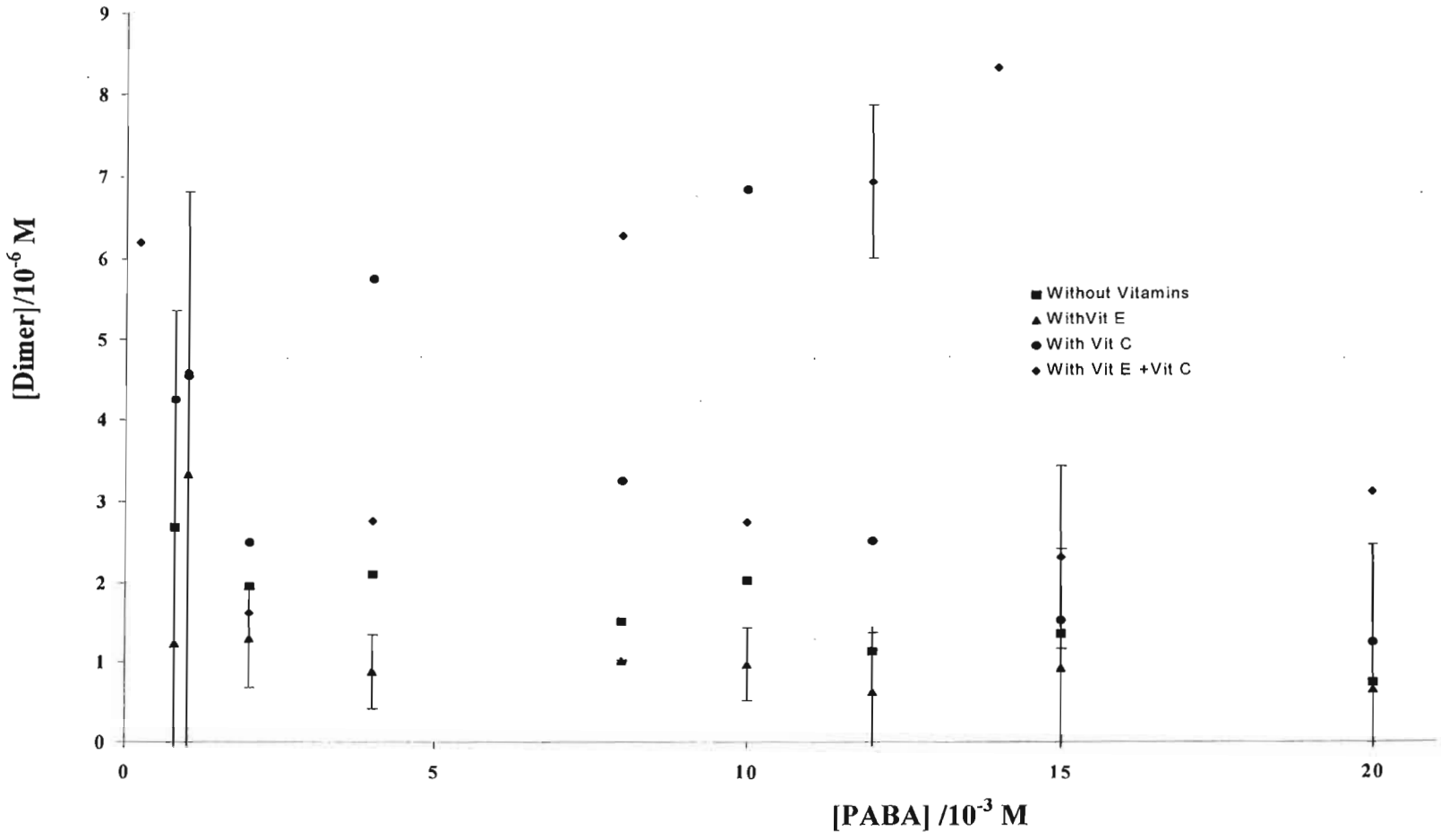

Figure: 3.8a: Thymine dimer formation as a function of PABA concentration at pH 3.0 and 6 hours irradiation, [thymine] = 1 x 10^{-2} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}. The error bars depict standard deviations for three replicate injections.

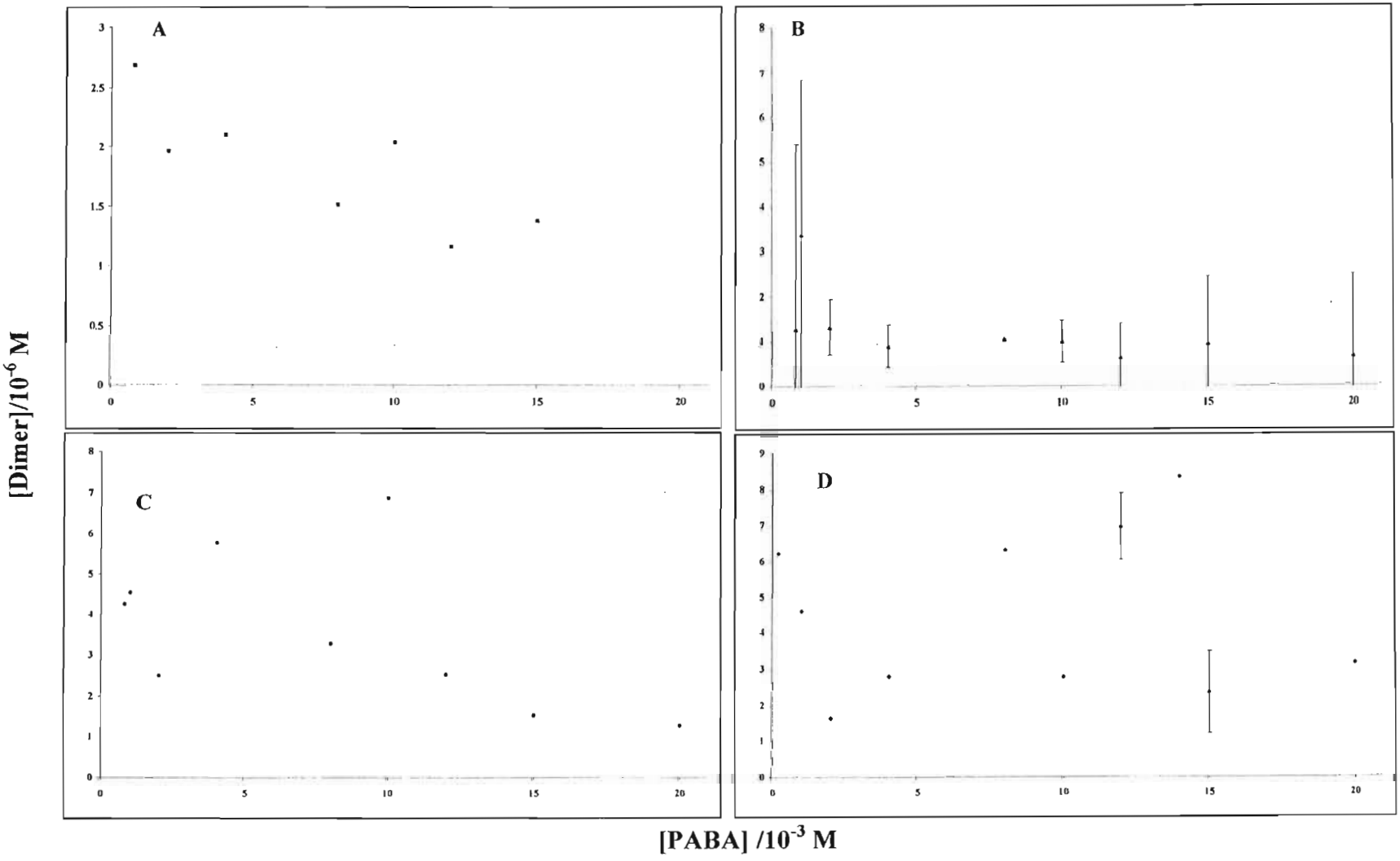

Figure: 3.8b: **A**, no vitamins added; **B**, vitamin E added; **C**, vitamin C added; **D**, both vitamins E and C added.

explanation is that in this situation, self-quenching becomes more important and that base stacking does not play a role at this pH. A typical chromatogram and the associated UV-spectra for this set of experiments are shown in Figure 3.9.

Addition of vitamin E decreases the dimer yield in this set of experiments. This observation contrasts with the observation on variable irradiation time for constant amount of vitamin E in which the dimer yield increased. In those experiments, apart from the 5-hour irradiation, the other irradiation times were longer than 6 hours. The decrease in dimer yield observed here can be explained in terms of the length of irradiation and the activity of vitamin E. A shorter irradiation time populates less triplet PABA and hence a smaller amount of the dimer is formed. Another plausible reason could be that the short irradiation time of 6 hours resulted in less destruction and side reactions of vitamin E leaving more of it to reduce the formation of dimer. As postulated earlier, vitamin could have quenched, reacted with triplet states of both thymine and PABA or reduce their lifetimes and thus reduce dimer yield. A typical chromatogram and the associated UV-spectra for this of experiments are shown in Figure 3.10.

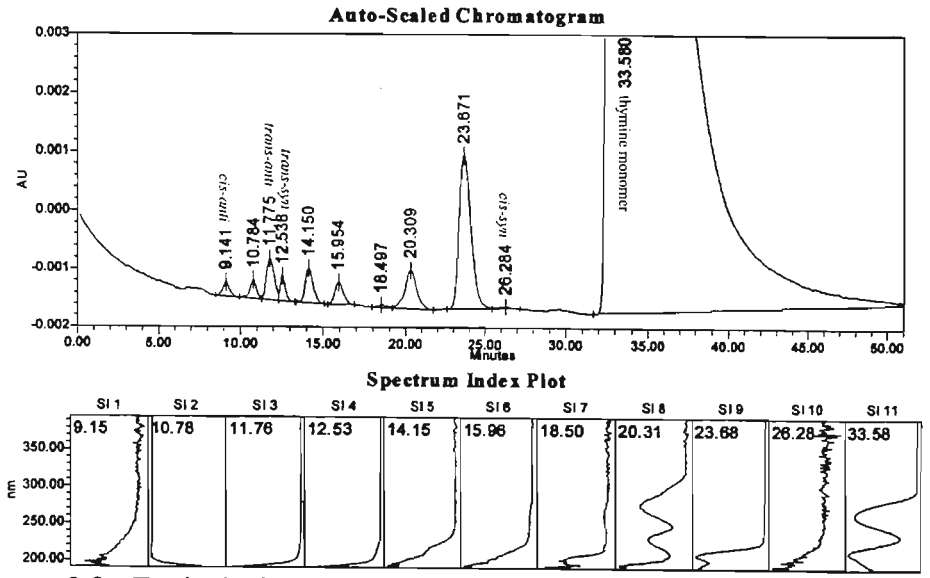

Figure 3.9: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M and [PABA] = 1 x 10^{-3} M at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

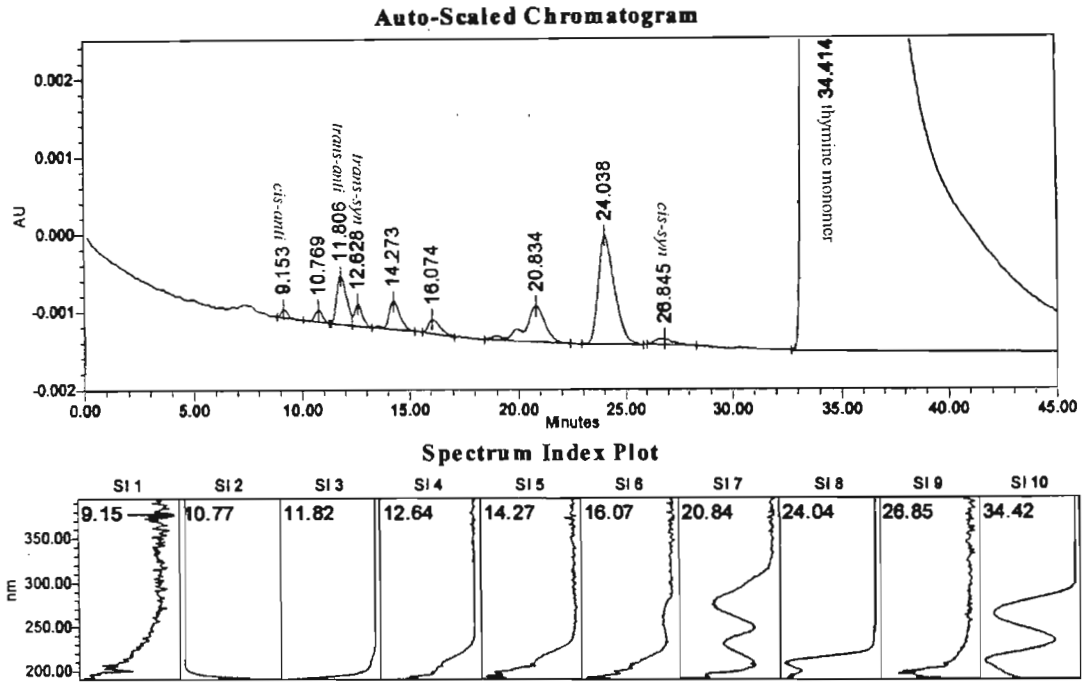

Figure 3.10: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 2 x 10^{-3}. M and [vitamin E] = 0.018 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

Vitamin E can provide protection against UV radiation by inhibiting the formation of cyclobutane pyrimidine photoproducts and especially thymine dimers **[154, 263]**. Although it is not clear how vitamin E does this, its antioxidant property is thought to be responsible for this inhibition of thymine dimer formation **[144]**. The chromatogram shown in Figure 3.10 shows no peak for vitamin E suggesting that there was no un-reacted vitamin E left after irradiation. Either it was all or partly consumed in lowering dimer yield and if there was any left after interacting with PABA and thymine then it was destroyed by UV radiation since it is known to be photolabile.

Addition of vitamin C increases the dimer yield. A possible explanation for this is that it is due to the increase in PABA concentration. As explained in Chapter 1 and by Equation 1.9, a photosensitiser can reduce the activity of a vitamin **[177]**. An increase in the concentration of PABA can reduce the activity of

vitamin C more than when there is a constant amount of PABA. Besides there will also be an increase in the number of PABA molecules populated to their triplet state and hence more dimer is formed. Although there are no references in the literature indicating a photoreaction between vitamin C and PABA, the increase in concentration of the latter might have caused a reaction that depletes the amount of vitamin C and hence reduced its activity. Another independent factor that could have resulted in the increase of dimer yield is the ability of vitamin C to cause DNA damage **[190]**. Jenkins *et al.* **[190]** showed that vitamin C alone at high concentrations has a prooxidant effect. It is believed that it can induce the formation of hydrogen peroxide and hydroxyl radical. These two radicals contribute to the total oxidative damage of DNA. Although they studied the oxidative damage caused by the antioxidant, there is a possibility that it can also increase thymine dimer yield. Figure 3.11 shows a typical chromatogram and the associated UV-spectra after addition of vitamin C and irradiation. The peak at 13.187 minutes represents vitamin C. Since there was no vitamin E to regenerate ascorbic acid from ascorbate ions, the peak is for un-reacted vitamin C. For the variable irradiation-time experiments, vitamin C lowered thymine dimer formation. The amount of vitamin C used in the variable time experiment and variable PABA concentration is the same. Therefore, a reasonable explanation for the increase in thymine dimer yield is that the photosensitising property of PABA seems to have outweighed the effect of vitamin C.

Introducing vitamin C and vitamin E together also has the effect of increasing dimer yield. Although synergism would have been expected to play a role here, the observation suggests otherwise. The most probable reason for this could be a combined prooxidant property of both vitamins i.e. antagonism. Figure 3.12 shows a typical chromatogram and associated UV-spectra for this set of experiments. The chromatogram does not show the presence of vitamin C or vitamin E yet the amounts used and irradiation times are the same when the two vitamins were added individually. Most likely, the un-reacted vitamin C that would have produced a peak at around 13 minutes has been consumed in regenerating vitamin E from its radical. We would once again have expected a peak for vitamin E but this is not the case. A possible explanation for this is

that consumption of vitamin E was the result of destruction by UV radiation to give many photoproducts **[144]**.

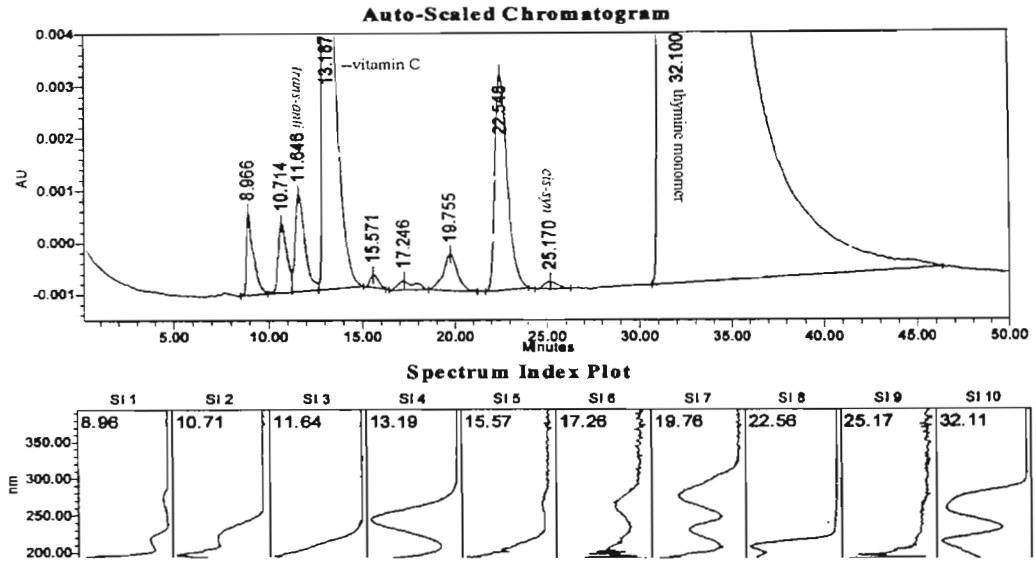

Figure 3.11: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 4 x 10^{-3} M and [vitamin C] = 0.044 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4 (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

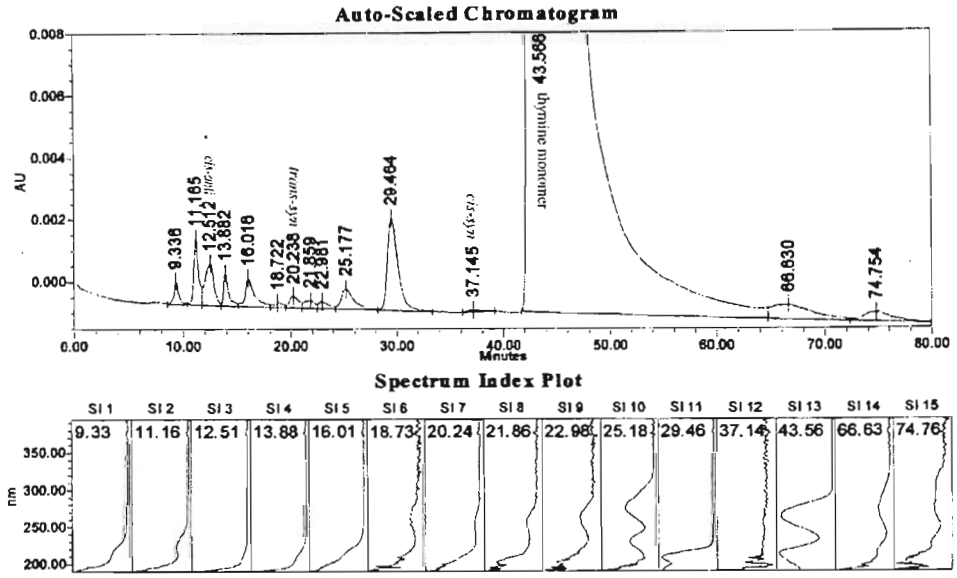

Figure 3.12: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 4 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 1% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

In this set of experiments in which the concentration of PABA was varied, baseline resolution of peaks was also achieved between individual dimers and also between dimers and other photoproducts. This is evident from the R values that are greater than 1.5 as summarised in Table 2.3. The order of elution for the thymine dimers in Figures 3.9 to 3.11 is the same as the one reported in Section 3.1.1 i.e. *cis-anti, trans-anti, trans-syn, cis-syn*. The presence of vitamins E and C did not change the order of elution as seen in Figure 3.10 and 3.11 respectively. Once again the introduction of the vitamins and PABA together resulted in many more different photoproducts. Separation also had to be achieved between any photoproduct and the dimer. To this end, a high resolution with R value greater than 1.5 between dimer and other photoproducts was achieved by using yet another set of chromatographic conditions i.e. a flow rate of 1.5 mL min^{-1} and 1% methanol in the case where both vitamins were present. The order of dimer elution changed to *cis-anti, trans-syn, cis-syn*, thymine monomer and lastly *trans-anti* dimer. This was confirmed by running standards of *cis-syn*, thymine and *trans-anti* as seen in Figure 2.30 in Chapter 2. The order of elution for the other two dimers was assumed to be *cis-anti* followed by *trans-syn*. As seen in Figure 3.12, *trans-anti* dimer was either not formed at all or if it was formed then it was not resolved from the thymine monomer peak. This had an effect on the quantitation of the *trans-anti*. In such a situation the value of the peak area was taken as zero. In this set of experiments, the *cis-syn* thymine dimer had the lowest percentage yield except in the experiments where vitamin E was used (See Figure 3.10). *Trans-syn* dimer had the highest percentage yield with only one exception when both vitamins were used (see Figure 3.12). *Cis-anti* dimer had the highest yield followed by *cis-syn* and lastly *trans-syn*.

3.1.3 EFFECT OF THYMINE CONCENTRATION

The following section discusses the data on thymine dimer yield obtained when the concentration of thymine was varied. Figure 3.13a shows dimer yield as a function of initial thymine concentration for initial constant concentrations of PABA, 1 x 10^{-3} M, vitamin E, 0.018 g L^{-1} and vitamin C, 0.044 g L^{-1} at pH 3.0 and 6 hours irradiation. Diagrams A, B, C and D in Figure 3.13b show the

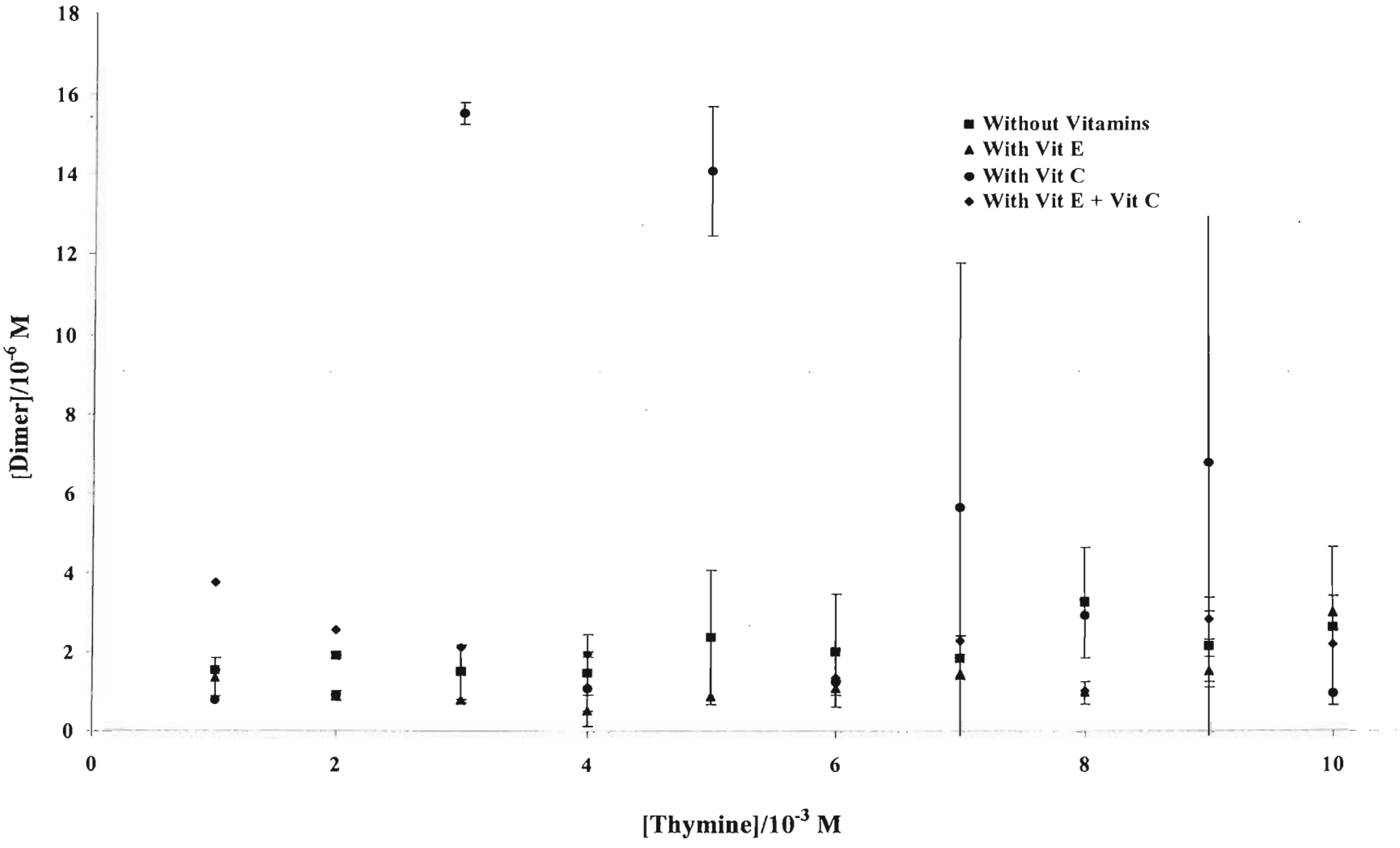

Figure 3.13a: Thymine dimer formation as a function of thymine concentration, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0 and 6 hours irradiation. The error bars depict standard deviations for three replicate injections.

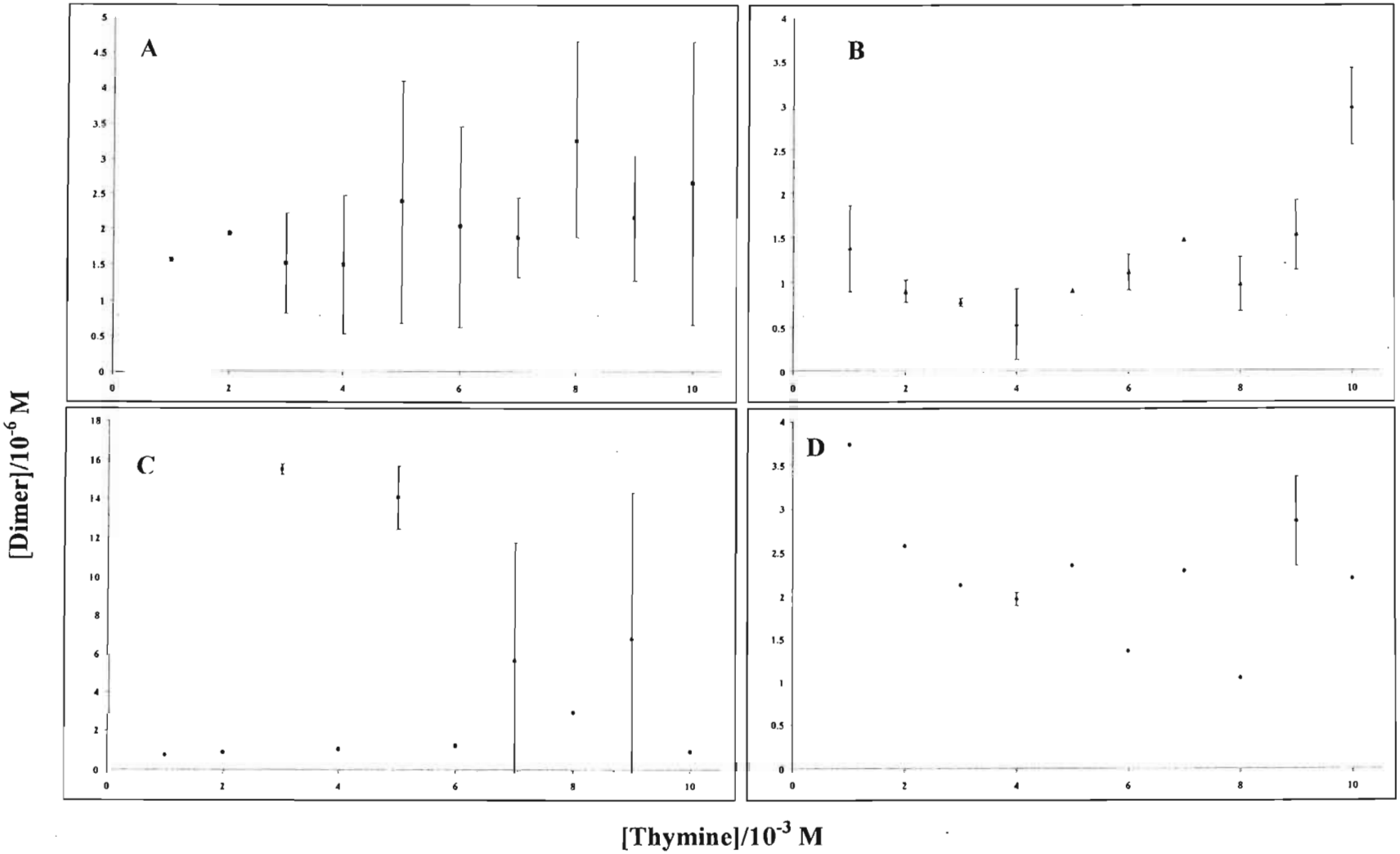

Figure 3.13b: : A, no vitamins added; **B**, vitamin E added; **C**, vitamin C added; **D**, both vitamins E and C added.

effect of absence of the vitamins, the presence of vitamins E, C and both vitamins respectively. Figures 3.14 to 3.17 show typical chromatograms and the associated UV-absorption spectra for the photoproducts obtained for these set of experiments.

Figure 3.14 shows the chromatogram and the associated UV-spectra for the irradiation of thymine and PABA alone. The concentration of thymine was 1 x 10^{-2} M but the chromatogram is typical of the set when the concentration of thymine was varied. Formation of unknown photoproducts other than the two dimers of thymine is evident as shown in the chromatogram. Both thymine and PABA **[85]** individually can produce photoproducts on irradiation as explained in Chapter 2 but it is not clear whether there are any photoproducts from chemical reaction between the two to the exclusion of light. In the absence of any antioxidant, there is a very slight increase in dimer yield with increase in initial thymine concentration. The graph is relatively constant compared to the other graphs, although the gradient is not very steep. The slight increase could be due to an increase in the amount of thymine, which increases the probability of reaction between ground state thymine and triplet PABA. An increase in collision frequency between triplet thymine and ground state thymine is also very likely. Another possibility is that with the given concentration of thymine increasing, base stacking could be a contributor. These factors can account for the increase in yield with an increase in thymine concentration.

Figure 3.15 shows a typical chromatogram and the associated UV-spectra for the irradiation of thymine, PABA and vitamin E. Addition of vitamin E alone significantly decreases dimer yield. The most likely reason for this decrease in the yield of the dimer could be that vitamin E quenches or reacts with the triplet states of both thymine and PABA, or reduces their lifetime. The triplets are necessary for the formation of the dimer. The results of our experiments agree with the findings by Krol *et al.* **[144]** that vitamin E can lower the formation of cyclobutane ring photoproducts and especially thymine dimers. They suggested that the sunscreen properties of vitamin E could play a role as much as the vitamin does not absorb significantly in the UVB region. Based on the premise of sunscreen properties, vitamin E could be absorbing some light energy that

would have otherwise been absorbed by the photosensitiser to promote the photosenstiser to its triplet state. Essentially, this results in a lower population of the triplet sensitiser that can collide with ground state thymine and thus resulting in low dimer yield. Some of the energy might also have been absorbed via the physical quenching as described in Equation 4 in Section 1.12 although the contribution would be very minimal. The chromatogram does not show the presence of vitamin E. This suggests that the vitamin was consumed in inhibiting the formation of thymine dimers and any un-reacted amount would have been destroyed by the irradiation.

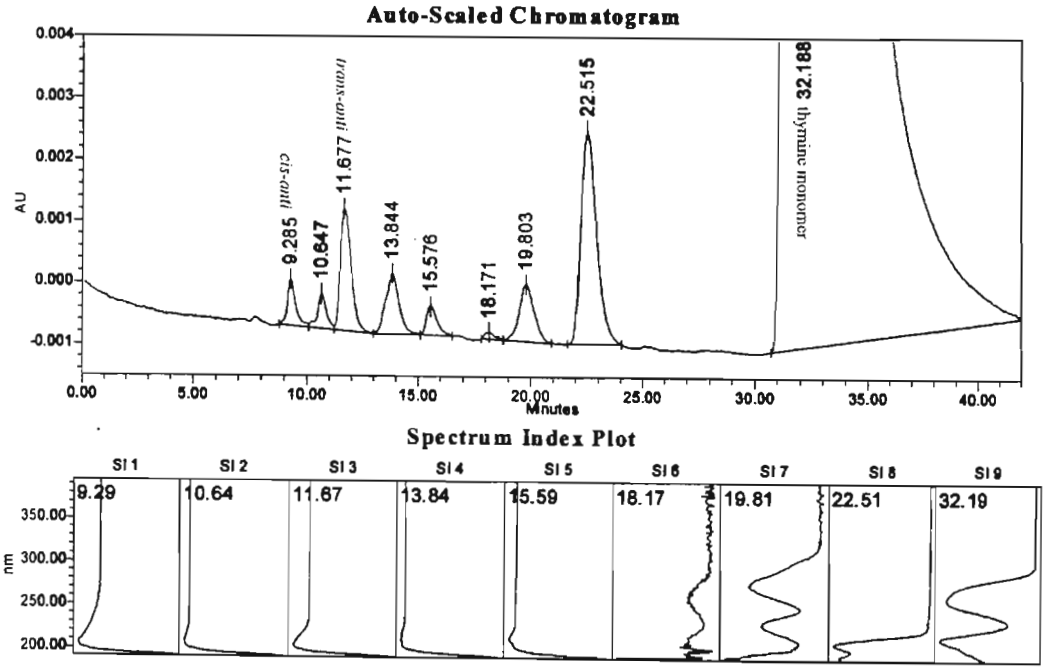

Figure 3.14: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M and [PABA] = 1 x 10^{-3} M at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

Figure 3.16 shows a typical chromatogram and the associated UV-spectra of photoproducts for the irradiation of thymine, PABA and vitamin C. Addition of vitamin C alone does not show a particular trend in dimer yield. Four out of the ten thymine concentrations studied showed an increase in dimer yield in no

particular sequence whereas six out of ten showed a decrease in dimer yield. An inference that can be drawn from this observation is that both pro- and anti-activities of the vitamin are taking part in the dimerisation process concurrently. The peak at 12.977 minutes in Figure 3.16 shows the presence of vitamin C. There was no vitamin E to regenerate Vitamin C therefore the presence of this peak is due to un-reacted vitamin C.

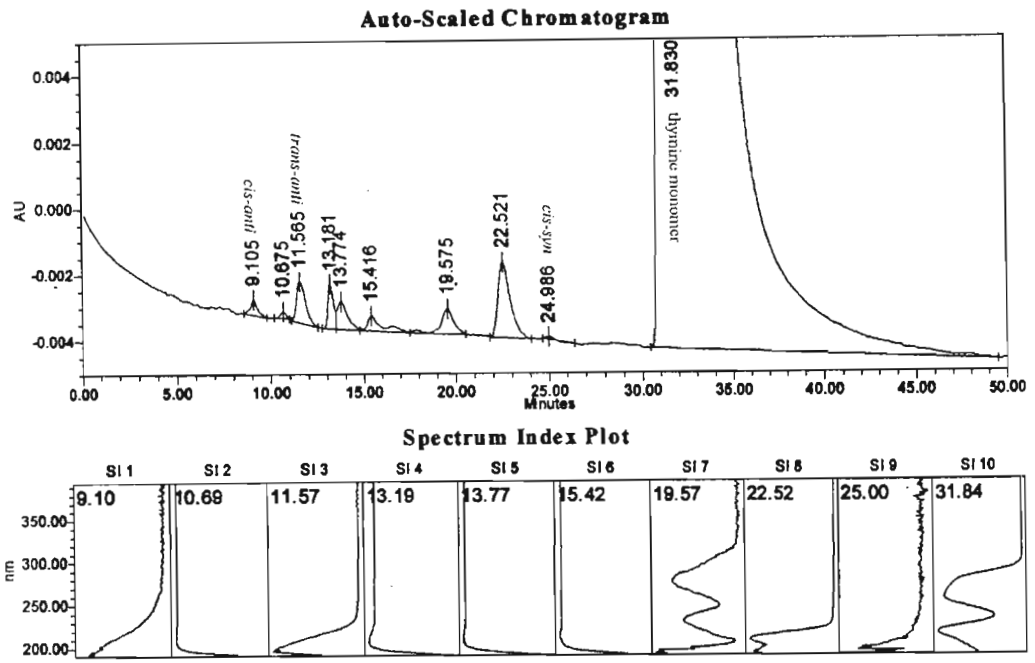

Figure 3.15: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin E] = 0.018 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

Figure 3.17 shows a typical chromatogram and the associated UV-spectra for the irradiation of thymine, PABA and the two vitamins. Addition of vitamin C and vitamin E together generally increases thymine dimer yield. This contrasts with the findings reported in most literature on the combined effect of the two vitamins on thymine dimer formation, which show that it can lower the formation of the dimer **[5, 86, 170]**. None of the studies reported by these researchers involved the use of a photosensitiser or variable concentrations of thymine. Therefore, this contrast can be explained in terms of a combined and

increased activity of the two vitamins. Vitamin E itself being a photosensitiser can contribute to the increase in dimer yield although this was not observed when it was present alone **[183]**. Prooxidant activity of the two vitamins increases with increase in interaction between the two **[177]**. Although there are no reports in the literature indicating any chemical reaction between the vitamins with either thymine or PABA, the possibility of photoproduct formation from a reaction between the vitamins and thymine and PABA cannot be ruled out. These could have photosensitising properties and hence increase thymine dimer formation. The chromatogram shows the presence of neither vitamin E nor vitamin C yet the same concentrations and chromatographic conditions were used as in Section 3.1.2. The most plausible explanation could be that the two vitamins were used in the lowering of formation of thymine dimer. Hence, this resulted in the two vitamins not being seen in the chromatogram.

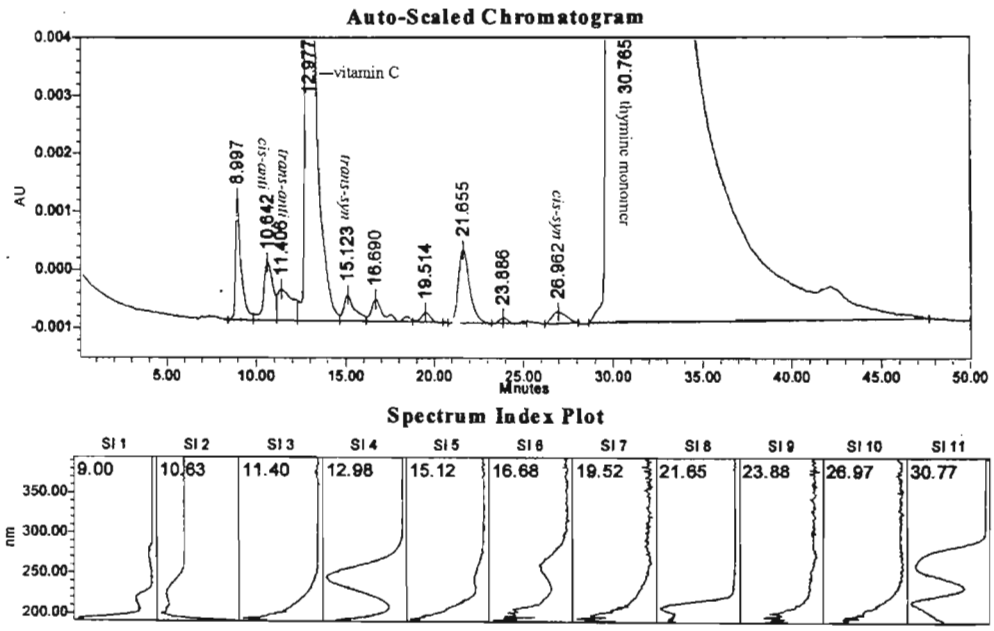

Figure 3.16: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin C] = 0.044 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

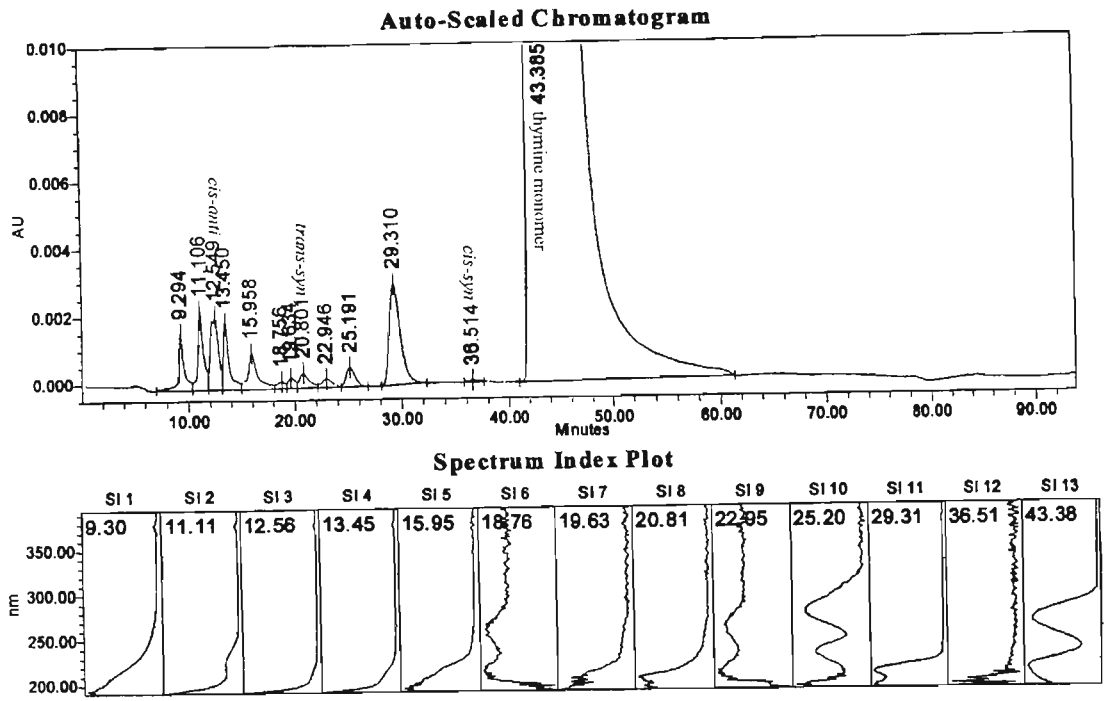

Figure 3.17: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 8 x 10^{-2} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

In this set of experiments in which the concentration of thymine increasing, baseline resolution of peaks was also achieved between individual dimers and between dimers and other photoproducts. The peaks for the photoproducts in the chromatograms in Figures 3.14, 3.15 and 3.16 were eluted by use of a flow rate of 1.5 ml min^{-1} and 4% (v/v) methanol. The order of elution is the same as for the one reported in Section 3.1.1, i.e. *cis-anti, trans-anti, trans-syn, cis-syn* and lastly thymine monomer. The presence of many different photoproducts necessitated new chromatographic conditions, i.e. 1.5 ml min^{-1} and 1% (v/v) methanol for the mobile phase. The order of elution is the same as the one reported in Section 3.1.2 for this condition, i.e. *cis-anti, trans-syn, cis-syn,* thymine monomer and lastly *trans-anti*. This was confirmed by running standards of *cis-syn,* thymine monomer and *trans-anti* dimer. For the other two dimers, the elution order was taken as *cis-anti* followed by *trans-syn*. There is no peak in this chromatogram to suggest the presence of *trans-anti* dimer.

Either it was not formed at all or if it was formed then it was not resolved from the thymine peak. Once again, the percentage yield for *cis-syn* is the lowest relative to the other dimers except for the set of experiments in which thymine, PABA and vitamin C were irradiated. *Trans-syn* dimer has the highest yield except for the set of experiments in which thymine, PABA and the two vitamins were irradiated. With few exceptions the order for the relative percentage yield is: *cis-syn* < *trans-anti* < *cis-anti* < *trans-syn.*

3.1.4 EFFECT OF VITAMIN E CONCENTRATION

The following section discusses the data on thymine dimer yield obtained when the concentration of vitamin E was varied. Figure 3.18 shows dimer yield as a function of initial vitamin E concentration for initial constant concentrations of thymine, 1 x 10^{-2} M, PABA, 1 x 10^{-3} M and vitamin C, 0.044 g L^{-1} at pH 3.0 and 6 hours of irradiation. Figures 3.19 and 3.20 show typical chromatograms and the associated UV-absorption spectra for photoproducts for this set of experiments.

Irradiation of thymine with PABA in the presence of vitamin E alone shows a slight linear increase in dimer yield but starts to level off at about 0.015 g L^{-1} of vitamin E. This implies that the amount of dimer formed remains generally constant. We would have also expected vitamin E to have a pronounced effect in lowering the yield of thymine dimer but the result does not support this. This unexpected phenomenon cannot be attributed to the initial concentrations of thymine and PABA since their concentrations are constant nor can it attributed to the irradiation time since it is still 6 hours. The only hypothesis to explain this is that, the increase in the amount of vitamin E increases the possibility of a chemical reaction between the vitamins with either thymine or PABA. This could result in photoproducts that have a photosensitising effect and hence can result in an increase in dimer formation for the first few concentrations. The levelling-off could be that the ability of vitamin E to lower thymine dimer formation is balanced by the formation of the photoproducts. From the chromatogram, there was a sufficient amount of thymine hence the number of

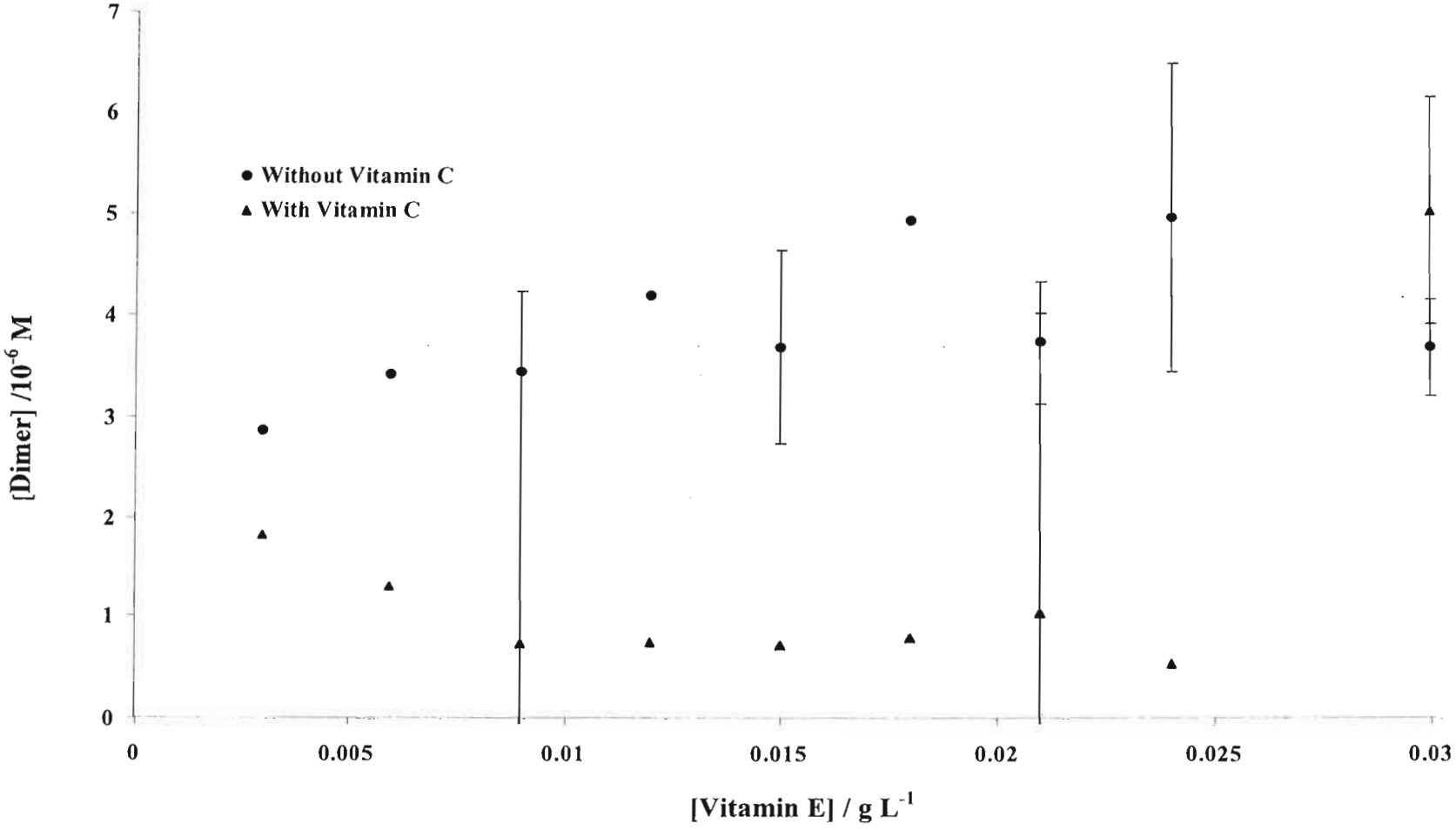

Figure: 3.18: Thymine dimer formation as a function of vitamin E concentration at pH 3.0 and 6 hours irradiation, [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin C] = 0.044 g L^{-1}. The error bars depict standard deviations for three replicate injections.

fruitful collisions between thymine and triplet PABA is maximum and hence could be another reason why there is an increase in yield. The chromatograms of the photoproducts suggest that there are other photoproducts besides the dimer. This is not surprising for PABA and thymine since they are known to give photoproducts in the presence of light **[85]**. A typical chromatogram and the associated UV-spectra of this set of irradiations are shown in Figure 3.19.

Addition of vitamin C substantially decreases dimer yield. As explained earlier vitamin C is capable of intercepting the excited triplet sensitiser and hence prevents it from interacting with ground state thymine **[177]**. Thus, vitamin C is able to lower dimer yield. A contributing factor is the synergism between the two vitamins. This has the effect of lowering the dimer yield. Although the formation of thymine dimer is lower in the presence of vitamin C, there is a decrease in the formation for the first three concentrations of vitamin E, i.e. 0.003, 0.006 and 0.009 g L^{-1}, and then the amount formed generally levels off. Again this implies the amount of dimer formed is essentially constant. A plausible explanation as to why it levels off lies on the properties of both vitamins. The ability of vitamin E to lower formation of thymine dimer is balanced by the formation of photoproducts that could have photosensitising properties. Vitamin C reduces the formation of thymine dimer and is thus depleted, leaving whatever effect that emanates from vitamin E as the dominant factor. A typical chromatogram and the associated UV-spectra for this set of experiments are shown in Figure 3.20.

For this set of experiments, baseline resolution of peaks was also achieved between individual dimers and between dimers and other photoproducts as seen in the two chromatograms. The peaks in the chromatogram in Figures 3.19 were eluted at 1.5 mL min^{-1} and 4% (v/v) methanol. The order of elution is the same as the one reported in Section 3.1.2, namely, *cis-anti, trans-anti, trans-syn, cis-syn* and lastly thymine monomer. For the chromatogram in Figure 3.20, the order of elution was *cis-anti, trans-syn, cis-syn,* thymine monomer and lastly *trans-anti* dimer. The peak at 14.178 minutes represents vitamin C. The

amount of vitamin C and irradiation time is the same as in the preceding cases. There was sufficient vitamin E to lower thymine dimer yield and also regenerate vitamin C. Therefore the peak showing the presence of vitamin C could be from two sources, i.e. un-reacted or regenerated. Although the concentration of vitamin E in the two chromatograms in Figure 3.19 and Figure 3.20 was increasing, there was no peak for unreacted vitamin E. This implies that all the vitamin was used to lower the dimer yield and whatever might have remained was destroyed by UV radiation. The peak at 13.542 minutes in Figure 3.19 is a split peak for the unknown photoproduct. In this set of experiments, there are only two sets of chromatograms to compare the peaks. In Figure 3.19, the order in the percentage yield of the dimers is *cis-syn* < *cis-anti* < *trans-anti* < *trans-syn*. In Figure 3.20, although only two dimers could be detected, comparison shows that *cis-syn* dimer still has the lowest percentage yield.

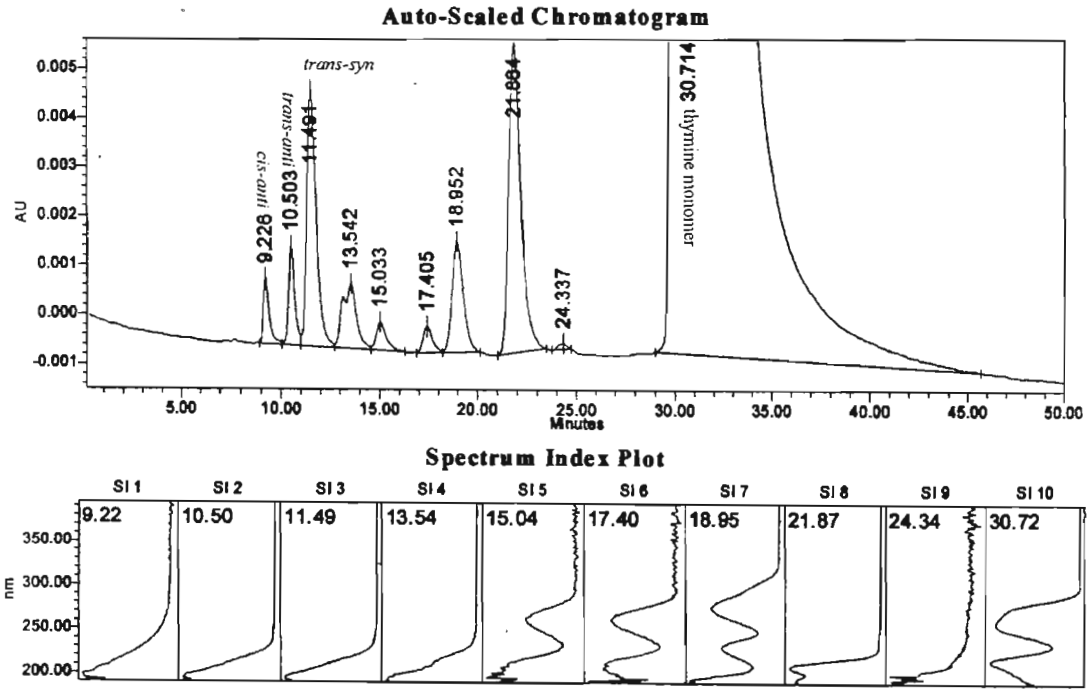

Figure 3.19: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin E] = 0.003 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 4% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

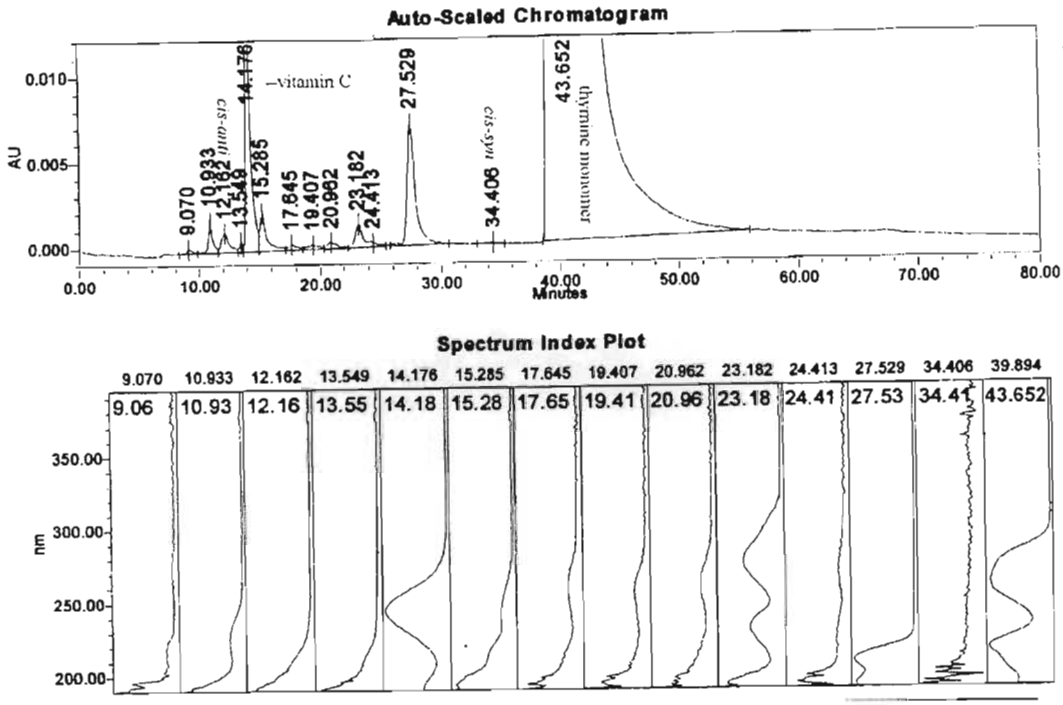

Figure 3.20: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 8 x 10^{-2} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.015 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 1% (v/v) methanol mobile phase with a flow rate of 1.5 mL min^{-1}. Detection was at 220 nm.

3.1.5 EFFECT OF VITAMIN C CONCENTRATION

The following section discusses the data on thymine dimer yield obtained when the concentration of vitamin C was varied. Figure 3.21 shows dimer yield as a function of initial vitamin C concentration for initial constant concentrations of thymine, 1 x 10^{-2} M; PABA, 1 x 10^{-3} M and vitamin E, 0.0180 g L^{-1} at pH 3.0 and 6 hours irradiation. Figures 3.22 and 3.23 show typical chromatograms and the associated UV-absorption spectra for photoproducts obtained for this set of experiments.

Irradiation of thymine and PABA with varying amounts of vitamin C shows an increase in dimer yield then a decrease. The increase can be attributed to the increase in prooxidant activity of vitamin C as its concentration increases. This

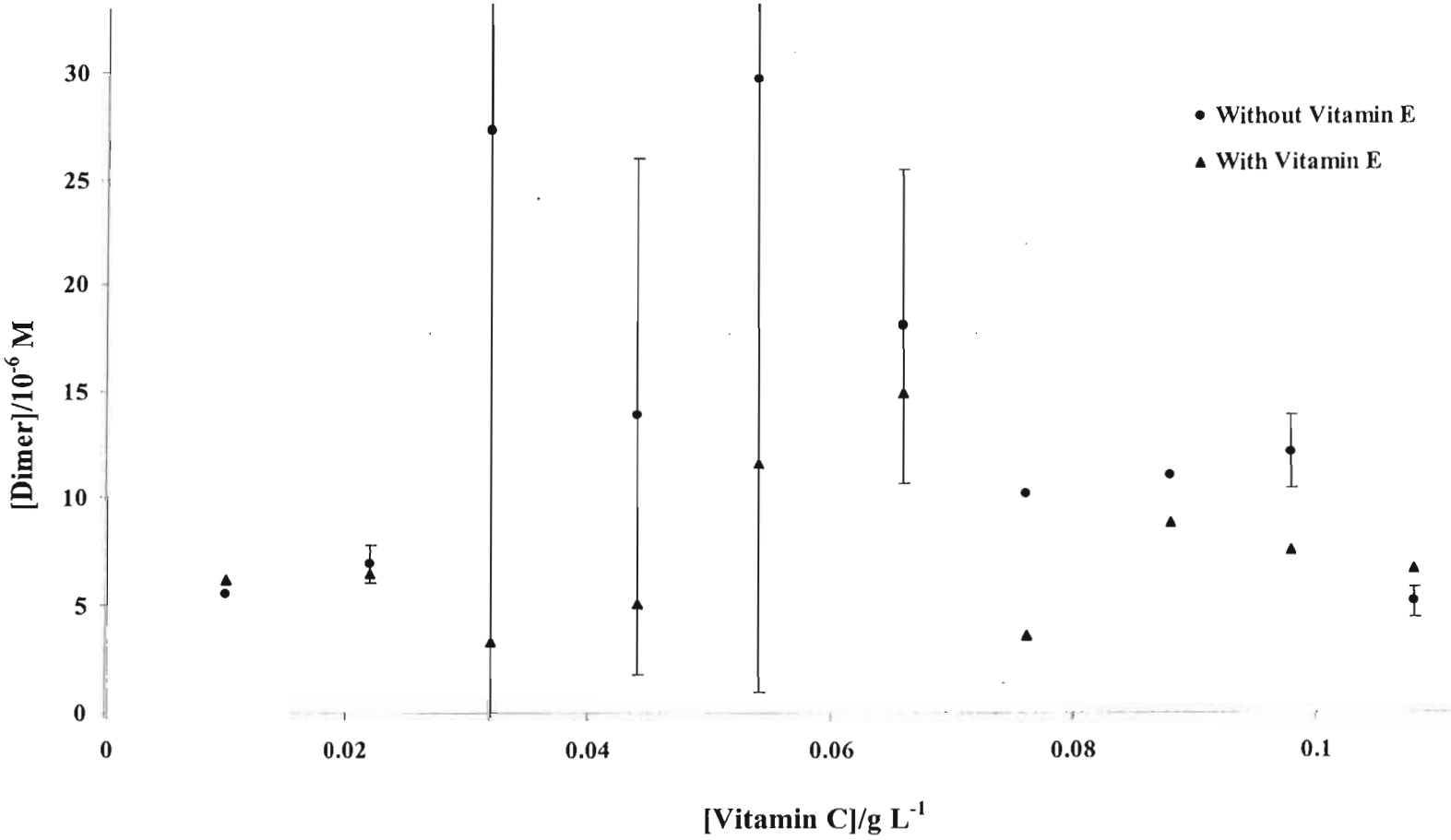

Figure: 3.21: Thymine dimer formation as a function vitamin C concentration at pH 3.0 and 6 hours irradiation, [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin E] = 0.018 g L^{-1}. The error bars depict standard deviations for replicate three injections.

is consistent with the findings by Jekins *et al.* **[190]** that vitamin C causes DNA damage at high concentration. They showed that at high concentration, vitamin C could increase the damaging effect of hydrogen peroxide that causes formation of DNA adducts and strand breaks. Although there have been no studies so far to suggest the negative effect of vitamin C regarding thymine dimer formation, there is a possibility that the same trend of adduct formation and strand breaks could apply to thymine dimer formation. Since the concentration of both thymine and PABA is the same, the only factor that determines the trend in dimer yield is the concentration of vitamin C. A probable explanation for the decrease after the increase is that there is a high concentration of vitamin C, enough to intercept triplet PABA and hence prevent thymine dimer formation. A typical chromatogram and the associated UV-spectra for this set of experiments are shown in Figure 3.22.

Addition of vitamin E significantly decreases dimer yield. This can be attributed to the synergism between vitamin C and vitamin E since both the vitamins are present. A typical chromatogram and the associated UV-spectra for this set of irradiations are shown in Figure 3.23.

The combined presence of vitamin E, vitamin C, thymine and PABA in this set of experiments also produced many photoproducts. Although a high resolution was achieved between dimers and thymine, the resolution between dimers and photoproducts was very poor and the runs were long with peaks of interest eluting after one hour since the flow rate was low. Despite the altered chromatographic condition of 0.9 ml min^{-1} and 1% (v/v) methanol, the order of elution remained the same as for the one obtained with a flow rate of 1.5 ml min^{-1} and 1% (v/v) methanol i.e. *cis-anti, trans-syn, cis-syn* and lastly thymine monomer. Standards of *cis-syn, trans-anti* and thymine as shown in Figure 2.29 indicated that *trans-anti* should elute at around 84.031 minutes but there is no peak at about 84 minutes to suggest the presence of *trans-anti* dimer. This implies that either it was not formed at all or it was not resolved from the thymine peak. There is no peak for vitamin E in the chromatogram in Figure 3.22 nor in the chromatogram in Figure 3.23. A plausible reason is that some of it was consumed by the thymine dimer yield and some was destroyed during

irradiation. The peak at 25 minutes in Figure 3.22 represents vitamin C. This is possibly a combination of un-reacted vitamin C portion and a portion that might have been regenerated from vitamin E. Such peaks for vitamin C also appeared at around 13 minutes for variable irradiation time, variable thymine and PABA concentrations, and at around 14 minutes for variable vitamin E concentration. In this set of experiments, there are only two chromatograms to compare the results. Nevertheless, the yield for *cis-syn* dimer was the lowest of the dimers.

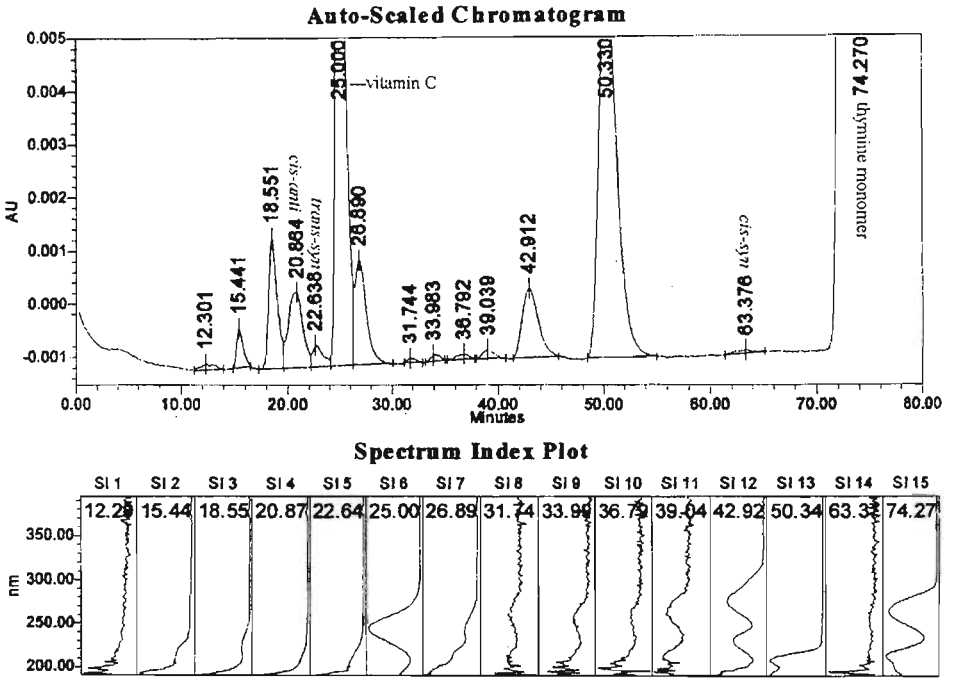

Figure 3.22: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [vitamin C] = 0.076 g L^{-1}at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 1% (v/v) methanol mobile phase with a flow rate of 0.9 mL min^{-1}. Detection was at 220 nm.

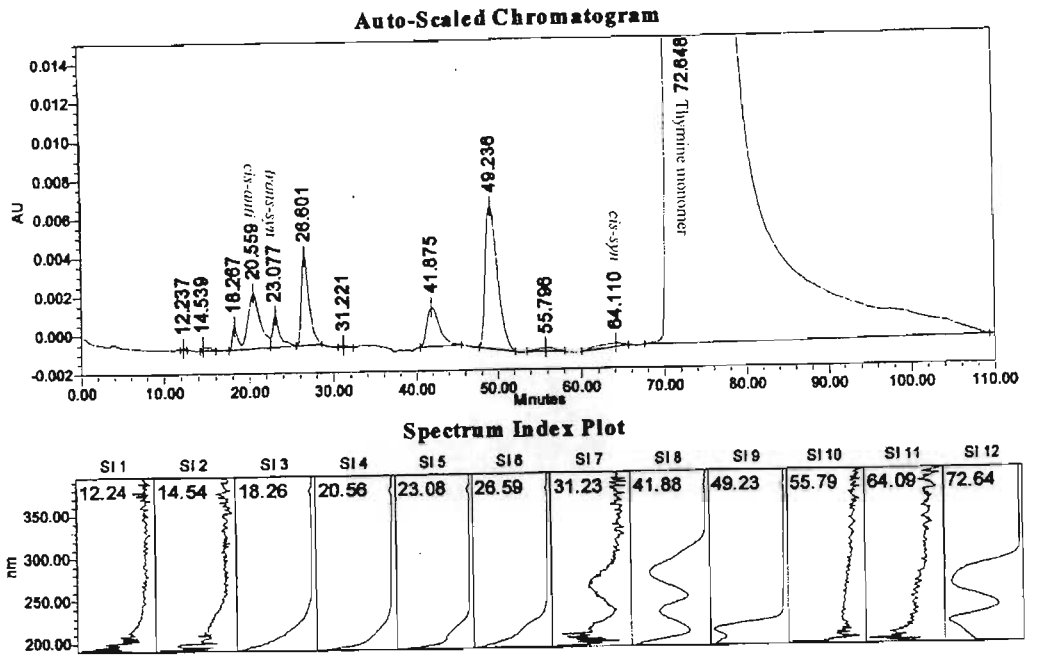

Figure 3.23: Typical chromatogram and spectral index plot for the photoproducts formed in a solution of [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.032 g L^{-1} at pH 3.0 irradiated for 6 hours and separated with an Ultracarb 5 ODS (20) column by using a 1% (v/v) methanol mobile phase with a flow rate of 0.9 mL min^{-1}. Detection was at 220 nm.

3.1.6 COMPARISON WITH CONTROLS

There were some specific observations noted in the spectra and chromatograms of both the experimental controls (see Section 2.8) and the samples that need some mention. Comparisons were drawn between the chromatograms of photoproducts of the controls and those of the photoproducts of the samples. This was to identify products that resulted from interaction of the reagent and not directly from the controls.

Generally, the spectra of the blanks showed weak absorption for the photoproducts formed apart from the peaks of un-reacted thymine, PABA and vitamin C, which had high absorption. This indicated that the amounts of products formed in the controls were of low concentration. From the thymine control shown in Figure 2.34, it can be seen that the photoproducts at 13.71 and 14.97 minutes are also formed when thymine and PABA are irradiated

together. The peak at 20.11 minutes (see Figure 2.34) does not appear in the irradiation that involves PABA and thymine. This implies that it is a photoproduct of thymine absorbing radiation. Irradiation of thymine and vitamin C shows no evidence of *cis-syn* or *trans-syn* thymine dimer. Irradiation of thymine and vitamin E gave photoproducts represented by peaks at 14.932 and 18.839 minutes as shown in Figure 2.39. These photoproducts are also seen in the irradiation of the following systems: thymine alone, thymine and PABA and thymine, PABA and vitamin E but are not seen in pure PABA or pure vitamin E. Therefore, they are from the absorption of radiation by thymine to the exclusion of other reagents. The peak at 12.76 minutes in the chromatogram in Figure 2.36 for the irradiation of vitamin E alone is not seen in the chromatogram in Figure 2.41, in which vitamin E and PABA were irradiated together. This implies that absorption of radiation by vitamin E gave different photoproducts from irradiation of PABA and vitamin E.

The following general conclusion can be inferred from the set of experiments and results obtained regarding thymine dimer formation. At pH 3.0, the formation of thymine dimer increases with increase in thymine concentration and with increase in irradiation time but decreases with increase in the concentration of PABA. The presence of vitamins C and E whether individually or combined affect the yield of thymine dimer formed by PABA-photosensitisation. Both vitamins can lower or increase the formation of thymine dimer. This depends on the irradiation time and concentrations of the vitamins, thymine and PABA and whether the two vitamins are present together or are present individually. Therefore, both synergism and antagonism between the two vitamins is exhibited with regard to thymine dimer formation. It is quite possible that the unidentified photoproducts play a role in the proposed mechanism. Therefore, their presence may affect the thymine dimer yield. The concentration of vitamin C was 2.5×10^{-4} M whereas that of vitamin E was 3.8×10^{-5} M. Clearly there was more vitamin C than vitamin E. Nevertheless, the difference in their amount cannot account for the observed difference between the effects of the two vitamins. Generally, the yield for the formation of *cis-syn* dimer was lowest whereas that of *trans-syn* was the

highest. The vitamins did not have any effect on the percentage yield of individual isomers.

3.2 PABA-PHOTOSENSITISED FORMATION OF SINGLET OXYGEN

The following sections discuss and present data that show trends in singlet oxygen formation for different variables. The data obtained from the measurements on singlet oxygen are given in Appendix D. The differences in absorbance between un-irradiated samples and irradiated samples have been plotted against either the concentration of the particular reagent being varied or irradiation time. The discussion is divided into two sections. The first section discusses PABA-photosensitised formation of singlet oxygen with respect to irradiation time, concentrations of the reagents and the effect of introducing the vitamins. The second section describes the observations regarding singlet oxygen formation in the presence of thymine. All the solutions were maintained at pH 3.0. The amount of singlet oxygen formed was inferred from absorbance values derived from the bleaching of RNO.

As mentioned in Chapter 2, absorbance measurements for samples in which vitamin C was present were all corrected for the bleaching of RNO by vitamin C. Absorbance measurements were also taken at 284 nm, the maximum absorption for PABA at pH 3.0 For all the systems in which PABA concentration was constant, the change in absorbance at 284 nm was constant. This fact indicates that there was no chemical change that involved PABA and hence, it was not consumed. Thus PABA's role was to transfer energy to molecular oxygen and form singlet oxygen. It also absorbs photons that would be absorbed by other species in the solution. The results in this section are discussed in the light of the quenching properties of the two vitamins, the photosensitising properties of PABA and the effect of thymine in the formation of singlet oxygen.

3.2.1 EFFECT OF IRRADIATION TIME

Figure 3.24 shows graphs of the change in absorbance measured at 350 nm against irradiation time for the following constant concentrations of reagents: [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0.

The change in absorbance increases monotonically with increase in irradiation time for the three sets of results, namely, the set in which there are no vitamins, the one in which vitamin C is present and the third one in which vitamin E is present. This observation indicates that the formation of singlet oxygen increases with increase in irradiation time monotonically. Linetsky *et al.* **[145]** used RNO to measure the quantity of singlet oxygen formed by UVA irradiation of ascorbic acid-glycated lens protein at pH 7 maintained by a phosphate buffer. Although they did not work at pH 3, they also found that an increase in formation of singlet oxygen with an increase in irradiation time. This can be explained in terms of the properties of triplet PABA. Triplet PABA with energy higher than singlet oxygen is formed with a rate constant of 1.6 x 10^{9} M^{-1} s^{-1} **[65]**. This, with the fact that triplet PABA has a long lifetime means prolonging the irradiation time increases its population and hence it has a greater probability of transferring its energy to molecular ground state oxygen. Thus, more singlet oxygen is formed and the graphs show an increase with irradiation time. A different trend is observed when the two vitamins are introduced together. Here, singlet oxygen formation increases for the first six minutes with increase in irradiation time. Thereafter, the change in absorbance levels off despite an increase in irradiation time. The part of the graph that is level indicates that rate of formation of singlet oxygen is constant. The most probable explanation for the second portion is that the antioxidant synergistic effect of the two vitamins balances the effect of the increase in triplet PABA and hence, the net result is a steady-state formation of singlet oxygen. The graphs that depict the presence of the vitamins, either individually or together, are lower than the graphs depicting no vitamin present. This implies that for the same conditions the total amount of singlet oxygen formed has been lowered by the presence of the vitamins either individually or together. Here, the total

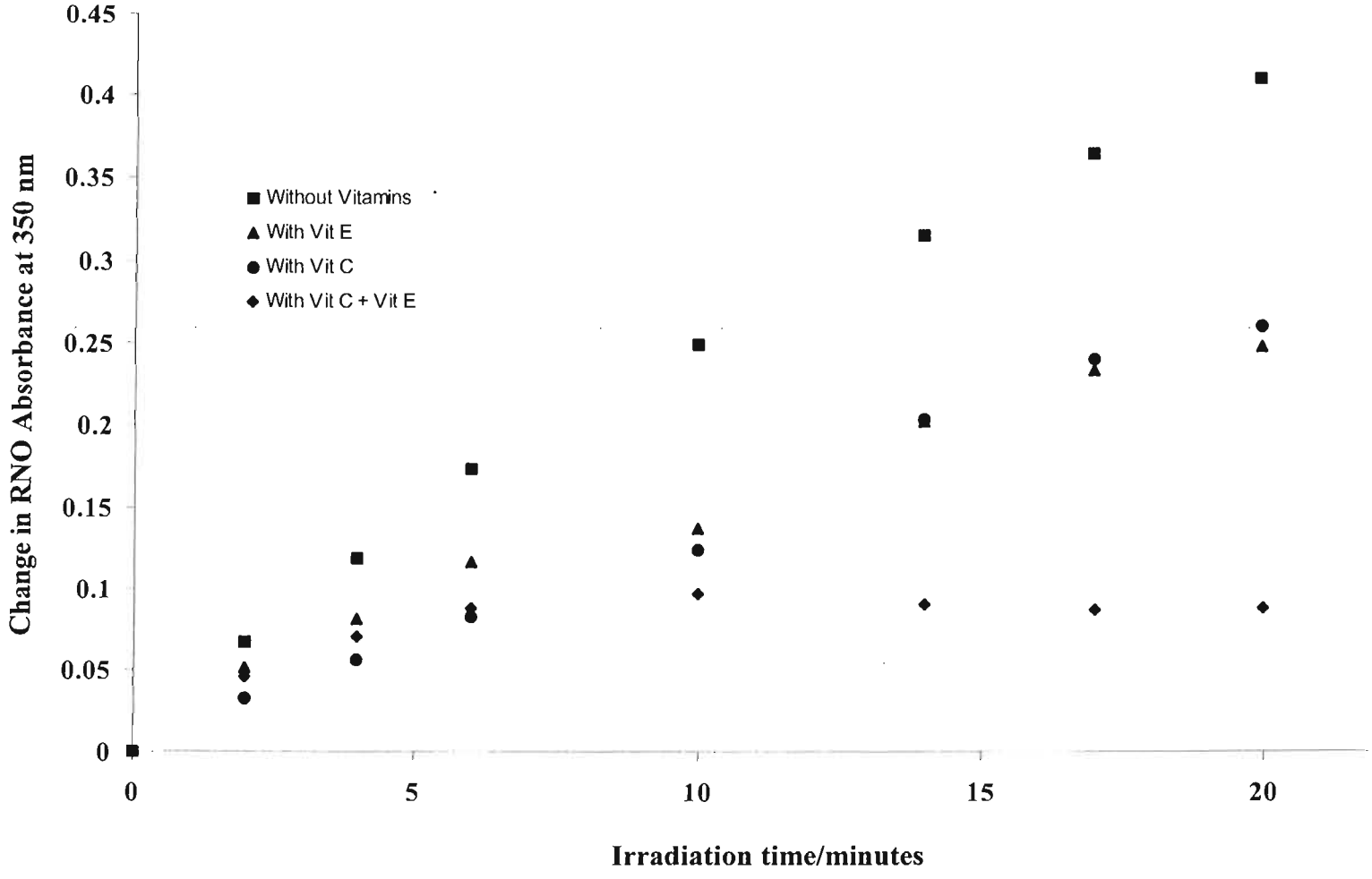

Figure 3.24: The effect of irradiation time on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm. The solution contained [RNO] = 4×10^{-5} M, [imidazole] = 8×10^{-3} M, [PABA] = 1×10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0.

effect of antioxidant activities, both individually and synergistically is much greater than the effect of PABA absorbing radiation to form an excited triplet state and photosensitise the formation of singlet oxygen. Hence, a smaller total amount of singlet oxygen is formed. For the first 14 minutes, vitamin C lowers the total amount of singlet formed. It does so more than what vitamin E does. This is not surprising since the concentration of vitamin C is 2.50×10^{-4} M whereas that of vitamin E is 3.81×10^{-5} M. After 14 minutes and within experimental error, the action of vitamin E is essentially the same the action of vitamin C, i.e. there is an increase in the formation of singlet oxygen with an increase in irradiation time. This phenomenon can be attributed to an increase in the population of triplet photosensitiser which overshadows the activities of the vitamins. After ten minutes, a combination of the two vitamins is more effective in quenching singlet oxygen than individual vitamins.

3.2.2 EFFECT OF PABA CONCENTRATION

Figure 3.25 shows graphs for the changes in absorbance measured at 350 nm against a changing concentration of PABA for a constant irradiation time of 15 minutes and the following constant concentrations of reagents: [imidazole] = 8×10^{-3} M, [RNO] = 4×10^{-5} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

As shown by the graphs, formation of singlet oxygen increases almost monotonically with increase in PABA concentration for all the experiments considered. At pH 3, an increase in PABA concentration should result in an increase in self-quenching of triplet state of PABA as explained during photodimerisation thymine in Section 3.1.2. The yield in thymine dimer leveled off at around 8×10^{-5} M of PABA. Therefore, an increase in PABA concentration should also translate into less formation of singlet oxygen because there are fewer PABA-triplet molecules. At high PABA concentration, all the photons may have been absorbed so that further increase in PABA concentration has no effect on singlet oxygen formation. It is also possible that under these conditions dissolved molecular singlet oxygen might be depleted. The data gathered also suggest the same, i.e. the increase in the formation of

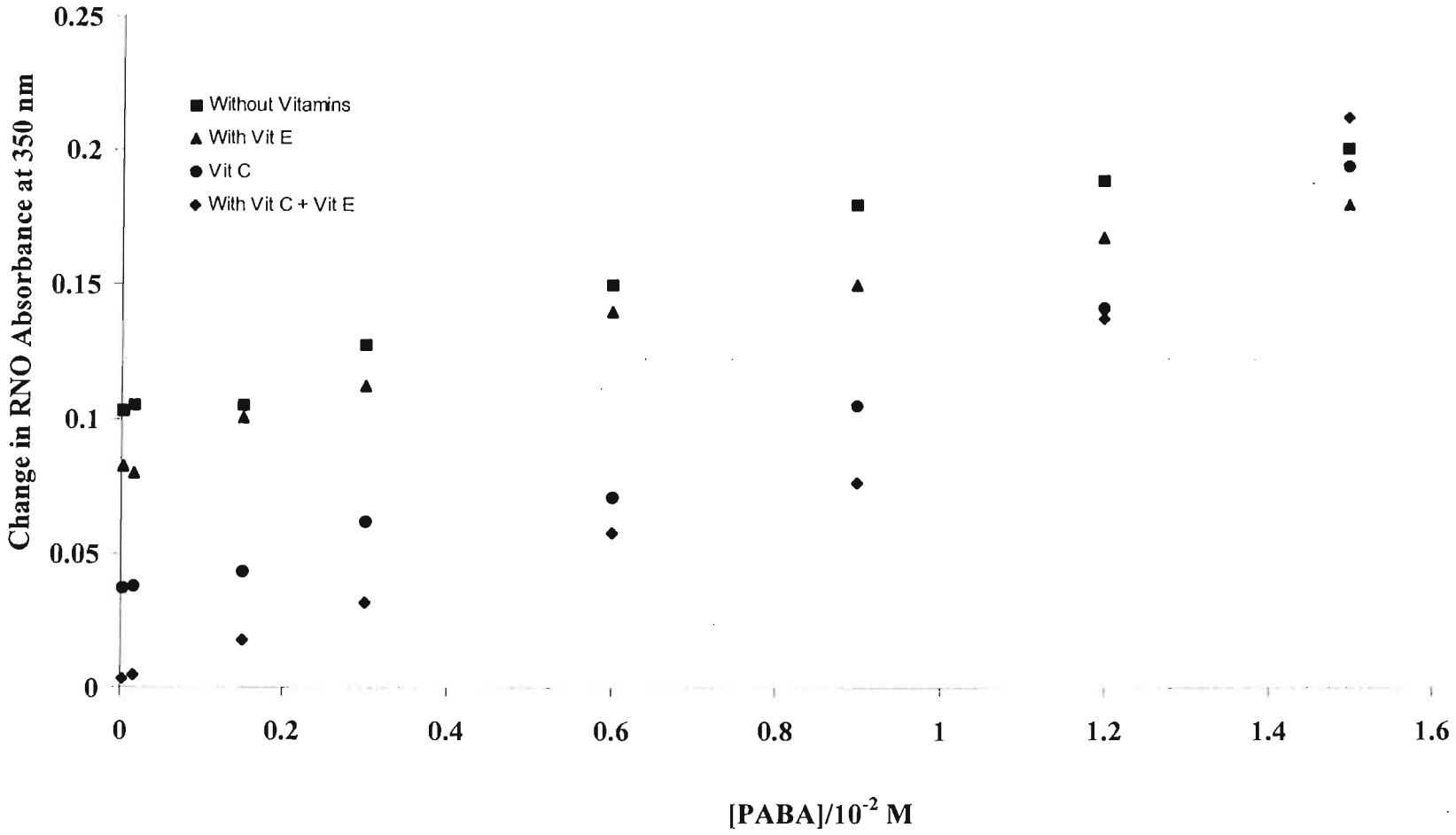

Figure 3.25: The effect of PABA concentration on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0.

singlet oxygen slows down at the higher concentrations considered. There is a plausible reason that can be advanced for this phenomenon and that is, at pH 3, the rate constant for self-quenching of PABA, 5 x 10^9 M^{-1} s^{-1} **[65]** (or 7.4 x 10^9 $M^{-1}s^{-1}$ **[74]**) is slower than the rate of formation of singlet oxygen by PABA-photosensitisation. Hence there is a state of balance between the two processes at high PABA concentrations and therefore the amount of singlet oxygen formed slows down. The increase in the concentration of PABA increases the total number of triplet PABA molecules but this is balanced with the increase in self-quenching of triplet PABA.

Both vitamins E and C and their combination reduce the amount of singlet oxygen formed by PABA-photosensitisation. The increase in order of quenching is vitamin E, vitamin C and lastly a combination of the two as the most effective. For this system, synergism between the two vitamins plays a role in reducing the formation of singlet oxygen. The fact that vitamin E is the least effective in quenching singlet oxygen can be attributed to its photosensitising properties **[183]**. For a PABA concentration greater than 1.5 x 10^{-2} M, the increase in order of quenching is a combination of the two vitamins, vitamin C and followed by vitamin E as the most effective quencher of singlet oxygen i.e. the order has been reversed. As explained in Chapter 1, photosensitisers can also reduce the activity of vitamins whether they are present individually or together. There is a possibility that the extent of this reduction in activity of the vitamins by a photosensitiser is different for different vitamins and hence the order changes. There is another possible reason as to why the amount of singlet oxygen formed in the presence of vitamin C is higher than that in the presence of vitamin E. This could be that vitamin C has been consumed to regenerate vitamin E whose activity has been reduced by the presence of the photosensitiser.

3.2.3 EFFECT OF THYMINE CONCENTRATION

Figure 3.26 shows graphs for the changes in absorbance, measured at 350 nm against changing concentration of thymine from 1 x 10^{-3} M to 1 x 10^{-2} M for a constant irradiation time of 15 minutes and the following constant

concentrations of reagents: [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [PABA] = 1 x 10^{-3} M. Three out of the four sets of results show a monotonic increase in singlet oxygen formation with increase in thymine concentration, namely, the set in which there are no vitamins at all and the two sets in which there are only individual vitamins. Similar trends were observed in the thymine photodimerisation. The concentration of PABA is constant throughout the range of thymine concentrations studied. Therefore, there cannot be an increase in triplet PABA to photosensitise singlet oxygen formation. The concentrations of the vitamins are also constant. Thymine does not absorb light in the region investigated. A plausible reason for this monotonic increase in the amount of singlet oxygen is the increase in PABA-photosensitised triplet thymine. As explained in Chapter 1, triplet thymine can react with molecular oxygen by either energy transfer or electron transfer to form singlet oxygen or superoxide radical ion respectively. There is no literature value for the rate constant for thymine-photosensitised formation of singlet oxygen in water. The quantum yields for formation of triplet thymine and singlet oxygen from triplet thymine in acetronitrile are 0.06 and 0.07, respectively **[101]**. The rate constant for PABA-photosensitised formation of triplet thymine in water at pH 3 is 1.4 x 10^{7} M^{-1} s^{-1} **[74]** (or 3.0 x 10^{7} M^{-1} s^{-1} **[65]**). The rate constant for the quenching of singlet oxygen by thymine is 2.5 x 10^{4} M^{-1} s^{-1} in 25% water and 75% DMSO **[103]**. The relative rate of formation of singlet oxygen by PABA to that of other sunscreen active ingredients at pH 8.5 in aqueous solution is quite high **[126]**. This property of PABA is attributed to the fact that it is a stronger absorber of radiation than thymine. This fact, together with the quantum yield of 0.07 for acetonitrile-photosenitised formation of singlet oxygen **[101]**, it is likely that PABA would form more singlet oxygen than thymine would under similar experimental conditions. From this data, there is also a strong indication that thymine-photosensitised formation of singlet oxygen is faster than quenching. Therefore, it will be reasonable to say that an increase in concentration of thymine results in an increase in more fruitful collision between thymine and PABA to produce more triplet thymine. This in turn, combined with the fact that both triplet PABA and triplet thymine photosensitise singlet oxygen formation results in an increase in the formation

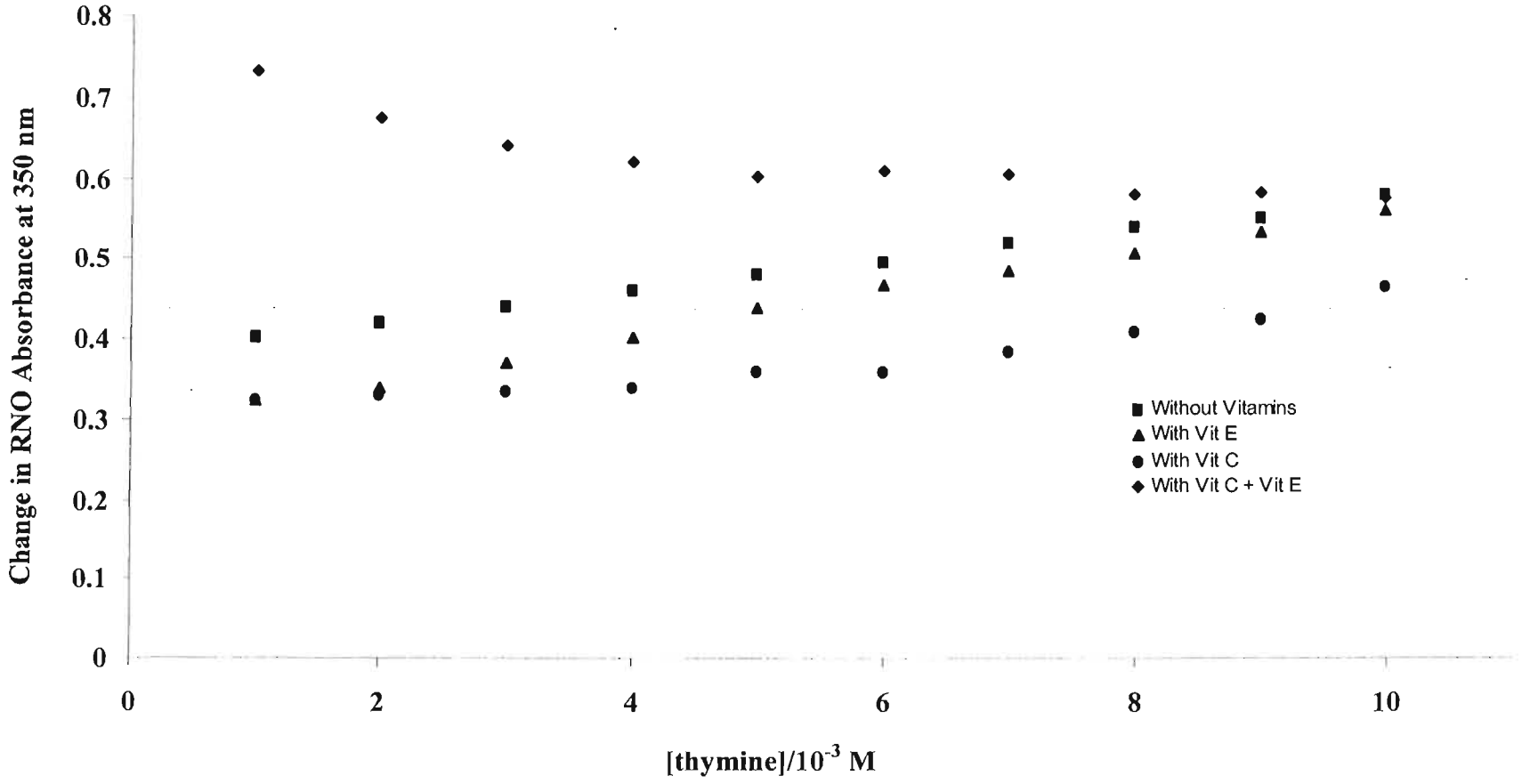

Figure 3.26: The effect of thymine concentration on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0.

of singlet oxygen as the thymine concentration increases. The total amount of singlet oxygen formed in the presence of both vitamins is more than in the absence of the vitamins. The only plausible explanation for this is that the combined prooxidant activity (antagonism) of the two vitamins is being exhibited here and that the vitamin E radical depletes vitamins in the regeneration. The amount of singlet oxygen formed in this experiment decreases with an increase in thymine concentration. This decrease is unexpected since an increase in thymine would result in an increase in population of the triplet state of thymine. An explanation for this phenomenon is that there could be a chemical reaction due to the presence of both vitamins that depletes thymine. As explained earlier in the paragraph, singlet oxygen formed also reacts with thymine **[103]** although slower than it is formed by thymine. With the amount of thymine decreasing, the number of triplet thymines necessary to photosensitise the formation of singlet oxygen consequently decreases. Hence, singlet oxygen formation decreases with increases in thymine concentration. The postulate that there could be chemical interaction between the vitamins and thymine needs further investigation. Individually, both vitamins C and E reduce the total amount of singlet oxygen formed with vitamin C being more effective than vitamin E. In the two cases, the amount formed increases with increase in the amount of thymine. The singlet oxygen scavenging properties of the two vitamins can explain the low amount of singlet oxygen formed. The increase in the formation of singlet oxygen with increase in thymine can be attributed to the increase in the population of triplet thymine. Physical quenching of the triplet photosensitiser by vitamin E (See Equation 4, Section 1.12) and interaction of vitamin C with the photosensitiser by a free radical mechanism (See equation 1.9, Section 1.13) could also contribute to a low population of the triplet states of both thymine and PABA. This implies that there are fewer triplets of thymine and PABA to photosensitise the formation of singlet oxygen. The order of quenching of singlet oxygen is a combination of both followed by vitamin E and lastly vitamin C being the most effective.

3.2.4 EFFECT OF VITAMIN E CONCENTRATION

Figure 3.27 shows graphs for the change in absorbance measured at 350 nm against variable concentration of vitamin E and a constant irradiation time of 15 minutes and the following constant concentrations of reagents: [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M and [vitamin C] = 0.044 g L^{-1}.

The formation of singlet oxygen in the absence of vitamin C is not affected by an increase in concentration of vitamin E. It is almost constant throughout the range considered. This could be due to a balance between quenching by vitamin E and its prooxidant activity with an increase in vitamin E concentration. Addition of vitamin C in this set of experiments lowers the total amount of singlet oxygen formed significantly. It also has the effect of increasing the formation of singlet oxygen monotonically as the concentration of vitamin E increases. The reduction in the total amount of singlet oxygen formed could be more due to the effectiveness of vitamin C regarding its antioxidant properties than due to the synergism between the two antioxidants. The monotonic increase could be due to depletion of vitamin C by the increase in concentration of vitamin E since vitamin C is depleted in the presence of vitamin E for the regeneration of the latter from its radical.

3.2.5 EFFECT OF VITAMIN C CONCENTRATION

Figure 3.28 shows graphs for the change in absorbance measured at 350 nm against the changing concentration of vitamin C for a constant irradiation time of 15 minutes. The following constant amounts of [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M and [vitamin E] = 0.018 g L^{-1} were used.

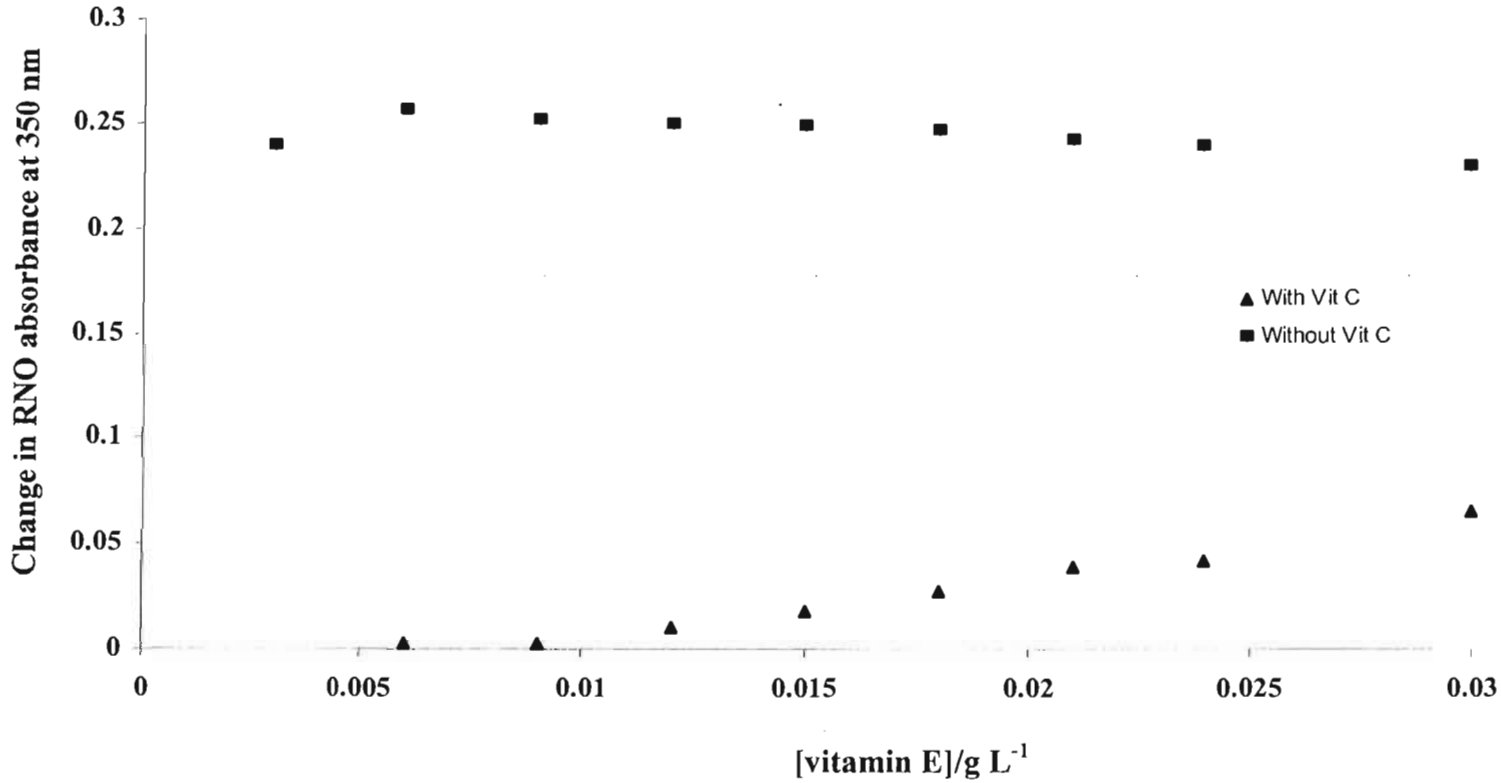

Figure 3.27: The effect of thymine concentration on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1} at pH 3.0.

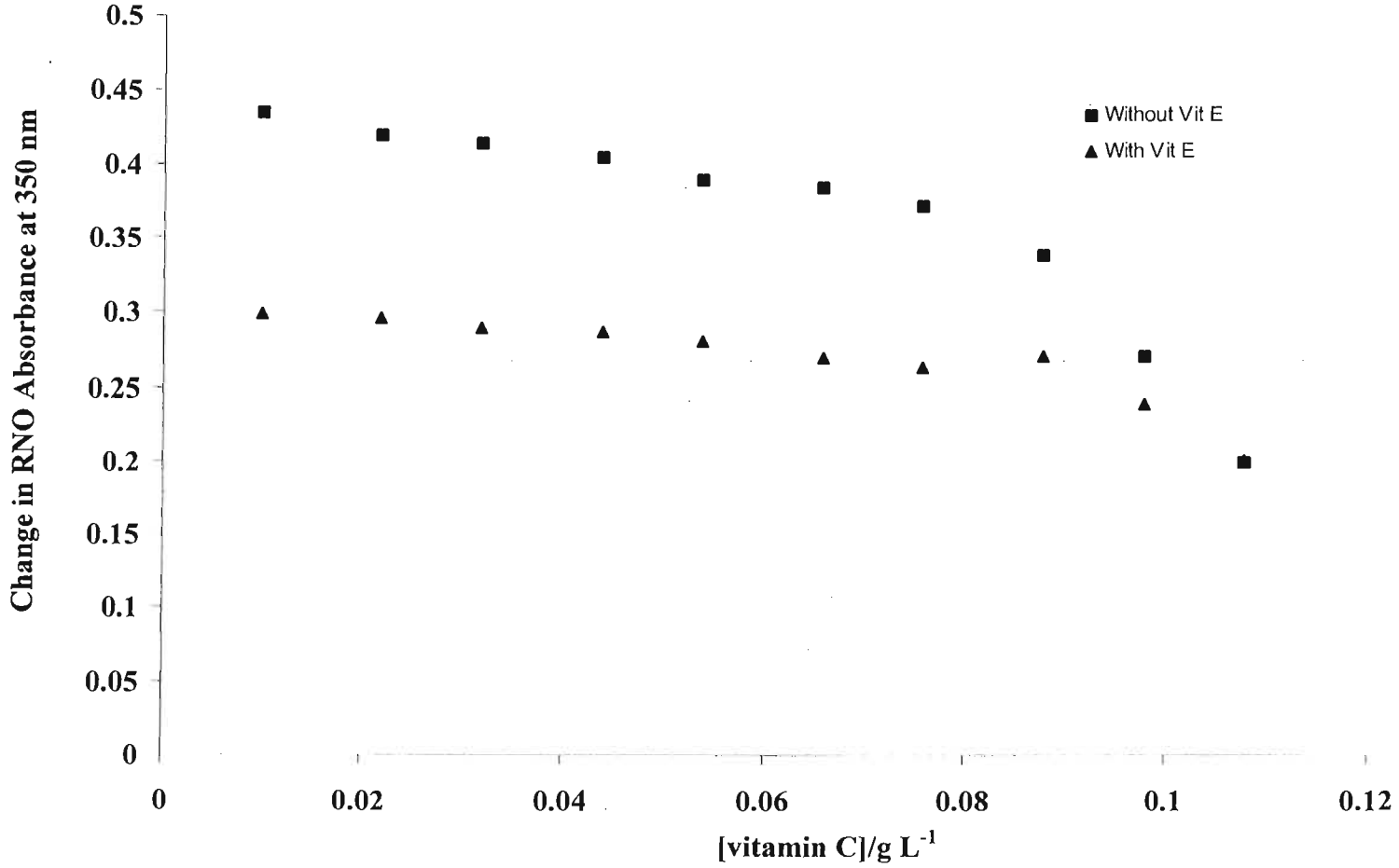

Figure 3.28: The effect of vitamin C concentration on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M and [vitamin E] = 0.018 g L^{-1} at pH 3.0.

The change in absorbance in both the cases considered decreases with increase in the concentration of vitamin C. In the set of experiments in which vitamin E is present, the decrease is linear. The set in which there is no vitamin E, the decrease is linear up to about 0.076 g L^{-1} of vitamin C, and then it starts to decrease more steeply. This implies that the total amount of singlet oxygen formed decreases with increase in vitamin C concentration. This means that at high concentration of vitamin C more singlet oxygen is scavenged. Thus vitamin C is a more effective antioxidant as its concentration increases. This agrees with the findings by Buettner *et al.* **[193]** that vitamin C. acts as an antioxidant in high concentration and as a prooxidant in low concentration. The increase in antioxidant activity could be due to more ascorbate ions being available to deactivate the energy of triplet PABA that is the precursor to singlet oxygen formation. Addition of vitamin E substantially reduces the total amount of singlet oxygen formed. This could be due to the combined effect (synergism between the two antioxidants). The presence of "excess" vitamin C enhances the regeneration of vitamin E from its radical

3.3 PABA-PHOTOSENSITISED FORMATION OF SINGLET OXYGEN IN THE PRESENCE OF THYMINE

The following sections present and discuss the results obtained from singlet oxygen measurements and the effect of adding thymine to the PABA-photosensitised reaction. It is also an effort to investigate the effect of thymine in the presence or absence of the two vitamins. Therefore thymine is contained in all the sets of experiments discussed below with its concentration constant at 1×10^{-2} M. The concentrations of all the other substrates were the same as those used in the previous section.

3.3.1 EFFECT OF IRRADIATION TIME

Figure 3.29 shows graphs for the changes in RNO absorbance measured at 350 nm against the irradiation time for the following constant concentrations:

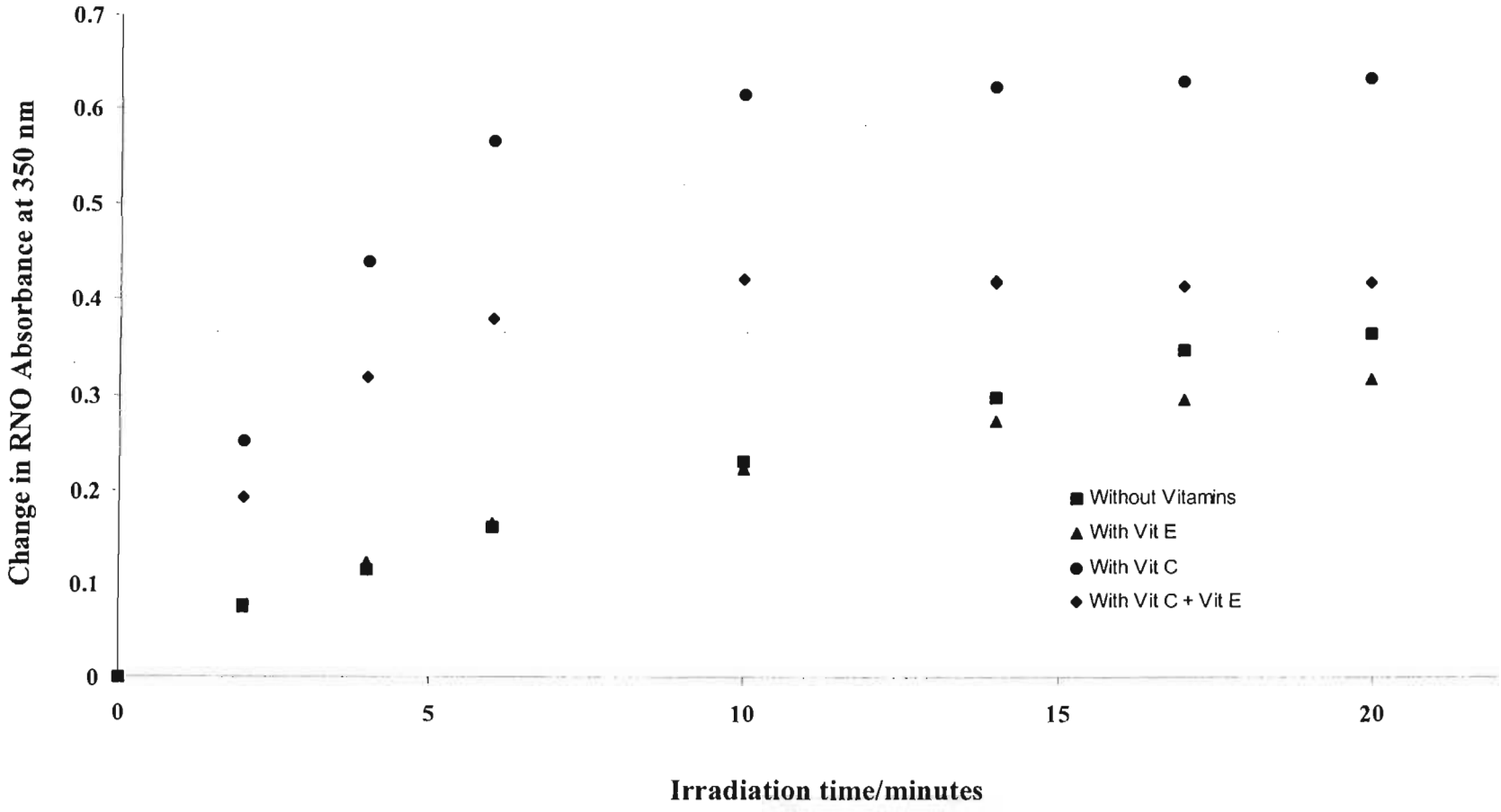

Figure 3.29: The effect of irradiation time on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M at pH 3.0.

[RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M.

The formation of singlet oxygen increases with an increase in irradiation time in all the sets of experiments considered. This can be explained in terms of an increase in the number of photons absorbed with an increase in irradiation time. At longer irradiation time more photons are absorbed resulting in a greater number of fruitful collisions. This leads to a greater probability of energy transfer to molecular oxygen to form singlet oxygen. The set of results that contain no vitamins, and the one that contains vitamin E only, show increasing formation of singlet oxygen with an increase in irradiation time. The addition of vitamin E does not affect the formation of singlet oxygen for the first six minutes. After six minutes vitamin E slightly decreases the formation of singlet oxygen. This is consistent with what Linetsky *et al.* **[145]** found with regard to irradiation time, i.e. the amount of singlet oxygen formed increases with increase in irradiation time (Section 3.2.1.) The set that contains vitamin C only and the set that contains both vitamins C and E show an increase in formation of singlet oxygen with an increase in irradiation time up to six minutes. Beyond six minutes, the graphs level off showing no observable change in singlet oxygen formation. Chemical quenching in the presence of vitamin C alone and in the presence of both vitamins could act to produce an opposite effect. It would be logical to expect an increase in triplet thymine and triplet PABA but chemical quenching could be reducing the number of thymine and PABA that can be populated to the triplet state. The two opposing forces balance and hence the amount of singlet oxygen remains constant and the graph levels. Addition of vitamin C or both vitamins C and E together has the effect of increasing the total amount of singlet oxygen formed. The constant concentration of vitamin used in this experiment was 0.004 g L^{-1} like in other sets. Consequently, this observation is unexpected for free vitamin C. Nevertheless, Linetsky *et al.* **[145]** used ascorbic acid glycated lens protein and found that it can form singlet oxygen. Therefore, a plausible explanation for high yield in the amount of singlet oxygen formed in the presence of vitamin C. This could be that vitamin C itself enhanced the formation of singlet oxygen for these experimental

conditions. In the latter case where vitamins C and E are combined, the phenomenon could be due to the combined prooxidant activity of the two vitamins (antagonism). The total amount of singlet oxygen formed in the presence of thymine and vitamin C alone is higher than in the presence of thymine and vitamin E alone or in the presence of thymine with the two vitamins together. In the presence of vitamin E, vitamin C is regenerated and synergism plays a role in its activity. This could explain the decrease in the total amount of detectable single oxygen formed. The order of quenching was vitamin C followed by a combination of the two vitamins and lastly vitamin E being the most effective.

Figure 3.30 combines the results shown in Figures 3.24 and 3.29. It compares the effect of the presence and absence of thymine in the photosensitized formation of singlet oxygen in the presence or absence of the two vitamins. All the graphs corresponding to the presence of thymine are above the respective graphs corresponding to the absence of thymine. Therefore, it is evident that thymine has the effect of increasing formation of singlet oxygen in the presence of vitamins whether the vitamins are combined or present individually. For a transient species like triplet thymine and triplet PABA, the induction time is short. Therefore there will be a sharp increase in the number of species for this period of time. After that, the rate of formation of the species is equal to the rate of it being consumed. Therefore a plot of its concentration versus times levels off after the induction period. The concentration of singlet oxygen will also show a similar pattern. Indeed the results from our experiments depict this phenomenon. Since in all the cases that involved thymine, the formation of singlet oxygen increased, there is a possibility of an additive effect in the sensitizing properties of thymine and PABA. The concentration of thymine in this set of experiments was 1×10^{-2} M whereas the amount of PABA was 1×10^{-3} M. These were the amounts used in the PABA-photosensitized dimerisation of thymine. Clearly there was enough PABA to populate triplet thymine and also to photosensitise the formation of singlet oxygen. Therefore the postulate on the additive effect seems more reasonable. One exception to this trend is the experiment that does not contain any vitamins or any thymine. The graph for this set is very slightly above the graph that corresponds to

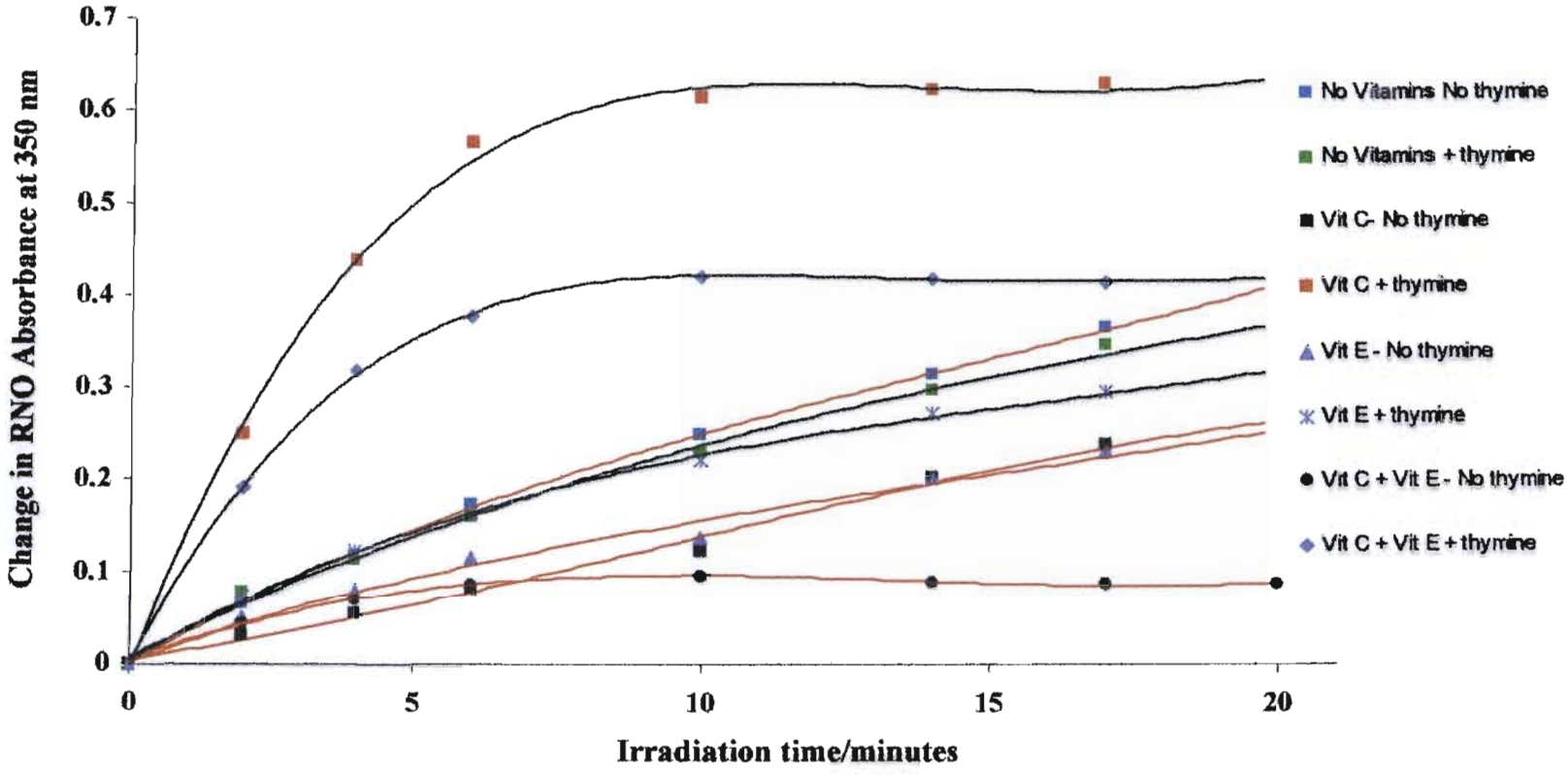

Figure 3.30: Comparison between the presence and absence of thymine and the effect of irradiation time and thymine on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm. The solution contained [RNO] = 4×10^{-5} M, [imidazole] = 8×10^{-3} M, [PABA] = 1×10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1×10^{-2} M at pH 3.0. The presence of thymine is denoted by black lines and its absence denoted by red lines.

the absence of vitamins but contains thymine. The two graphs diverge at around 10-minutes irradiation time with the graph that contains neither vitamins nor thymine being above the other. The graphs are very similar and the same explanation holds for the yield above 10 minutes as it does for less than 10 minutes, i.e. thymine photosensitises the formation of singlet oxygen.

3.3.2 EFFECT OF PABA CONCENTRATION

Figure 3.31 shows graphs for the change in RNO absorbance measured at 350 nm against PABA concentration for a constant irradiation time of fifteen minutes. The following reagent concentrations were constant: [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M.

The formation of singlet oxygen is almost constant with an increase in PABA concentration for the sets of results where no vitamins have been added and the set in which vitamin C has been added. For the experiments in which vitamin E was added, the formation of singlet oxygen is constant up to 6.0 x 10^{-3} M PABA, after this concentration, the formation of singlet oxygen increases with increase in PABA concentration. An increase the concentration of PABA results in an increase in the number of photons absorbed and hence more singlet oxygen being formed. There might have been some increase in self-quenching of triplet PABA but the increase in number of photons is more significant. Introducing vitamins C and E together has the effect of decreasing the formation of singlet oxygen as the concentration of PABA increases. This trend continues up to 6.0 x 10^{-3} M and thereafter any further increase has no effect on the formation singlet oxygen. This observation is consistent with the fact that at pH 3, an increase in PABA concentration results in an increase in self-quenching of triplet PABA. This translates into less formation of singlet oxygen because there are fewer triplets of PABA.

Vitamin E, in the presence of thymine has the effect of reducing the total amount of singlet oxygen formed but vitamin C in the presence of thymine has the

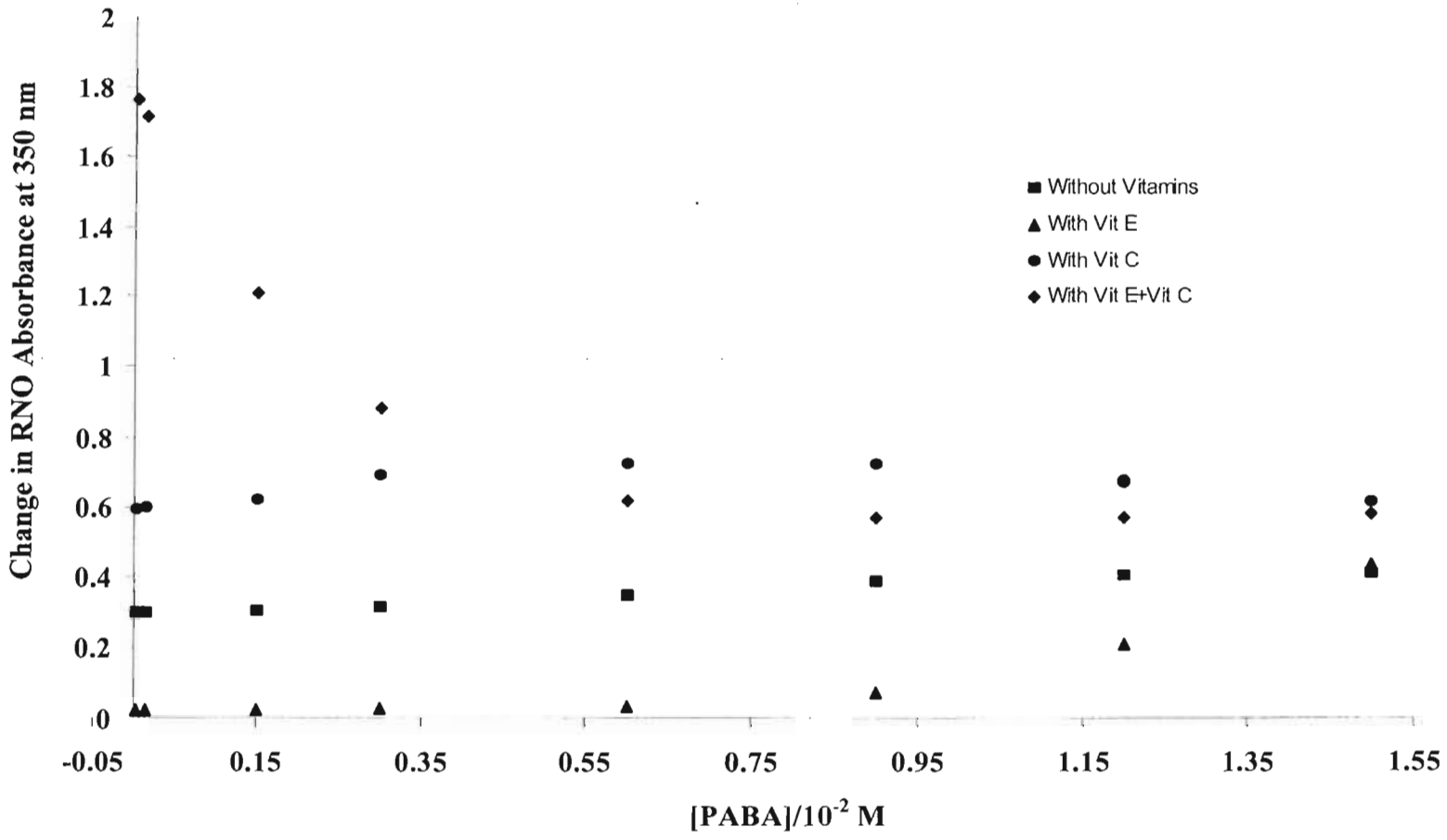

Figure 3.31: The effect of the concentration of PABA on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M at pH 3.0.

opposite effect. The reduction in singlet oxygen formation by vitamin E is not surprising. It is due to its antioxidant activity where it is able to quench singlet oxygen. Since the concentration of PABA is increasing, this fact together with thymine (a possible photosensitiser) could be reducing the ability of vitamin C to quench singlet oxygen **[177]** as described in Chapter 1. Vitamins E and C together have the combined effect of increasing the total amount of singlet oxygen formed compared to when there are no vitamins at all. The presence of both vitamins increases the formation of singlet oxygen more than does vitamin C alone up to about 4.5×10^{-3} M of PABA. Thereafter, vitamin C increases the formation of singlet oxygen more than does the two vitamins together. In the case of vitamin C, the high yield of singlet oxygen formed after 4.5×10^{-3} M of PABA could be attributed to a greater reduction in the activity of vitamin C with increasing PABA concentration. It is possible that the reduction in the activity of vitamin C is more than on the combined presence of the two vitamins. The observation that the graph representing vitamin C alone and the one representing the two vitamins show higher yield than in the absence of any vitamin can be attributed to prooxidant properties of vitamin C and the combined presence of the two vitamins. When combined, the two vitamins can exhibit an additive prooxidant property (antagonism). In this set of experiments, antagonism manifests itself in the total amount of singlet oxygen formed i.e. it is high. A striking observation is the trend in this total amount of singlet oxygen formed in the presence of both vitamins with an increase in PABA concentration. The trend show a steep decrease up to about 6×10^{-3} M, thereafter remains constant throughout. A plausible suggestion for this decrease in the formation of singlet oxygen is self-quenching of PABA as the concentration of PABA increases. A similar trend was observed with dimer yield and it is possible that self-quenching of triplet PABA is significant. It is not clear why the same phenomenon is not seen in the other set of experiments in which PABA concentration is also increasing. The order of quenching was vitamin C followed by a combination of both and lastly vitamin E being the most effective.

Figure 3.32 combines the results from Figures 3.25 and 3.31. It compares the effect of adding thymine on different sets of experiments. All the graphs for

experiments involving the presence of thymine are above those for absence of thymine with the exception of that which contains vitamins E alone. This implies that for a changing concentration of PABA, thymine in the presence or absence of vitamins has the effect of increasing the total amount of singlet oxygen formed. The possible photosentising property of thymine (as explained in the previous sections) in this system is the only plausible explanation for this observation. The graph representing vitamin E alone in the presence of thymine is below all the graphs representing the absence of thymine up to about 9.0 x 10^{-2} M and thereafter it is above all the graphs that represent the absence of thymine. This implies that as the concentration of PABA increases, vitamin E, in the presence of thymine reduces singlet oxygen formation up to a point and then the formation of singlet oxygen increases. For this set, the effect of the thymine photosensitizing property could be less than the effect of the antioxidant property of vitamin E up to 9.0 x 10^{-3} M in these experiments.

3.3.3 EFFECT OF VITAMIN E CONCENTRATION

Figure 3.33 shows graphs for the change in RNO absorbance measured at 350 nm against vitamin E concentration for a constant irradiation time of 15 minutes. The following concentrations were kept constant: [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M.

In the absence of vitamin C, the formation of singlet oxygen is constant with an increase in vitamin E concentration. Vitamin E is known to act as an anti-oxidant in low concentration and as a prooxidant at high concentration. This is not suggested by the trend of singlet oxygen formation as the concentration of vitamin E increases. We would expect to see a graph that shows a low yield of singlet oxygen at low vitamin E concentrations and a high yield at high concentrations. That the formation shows a steady production implies that there must be a balancing act between the two opposing effects and hence the net result is that there is a constant amount of singlet oxygen being produced. This balance could be due to the presence of thymine. As much as thymine has been shown to photosensitise the formation of singlet oxygen, it is also known to act

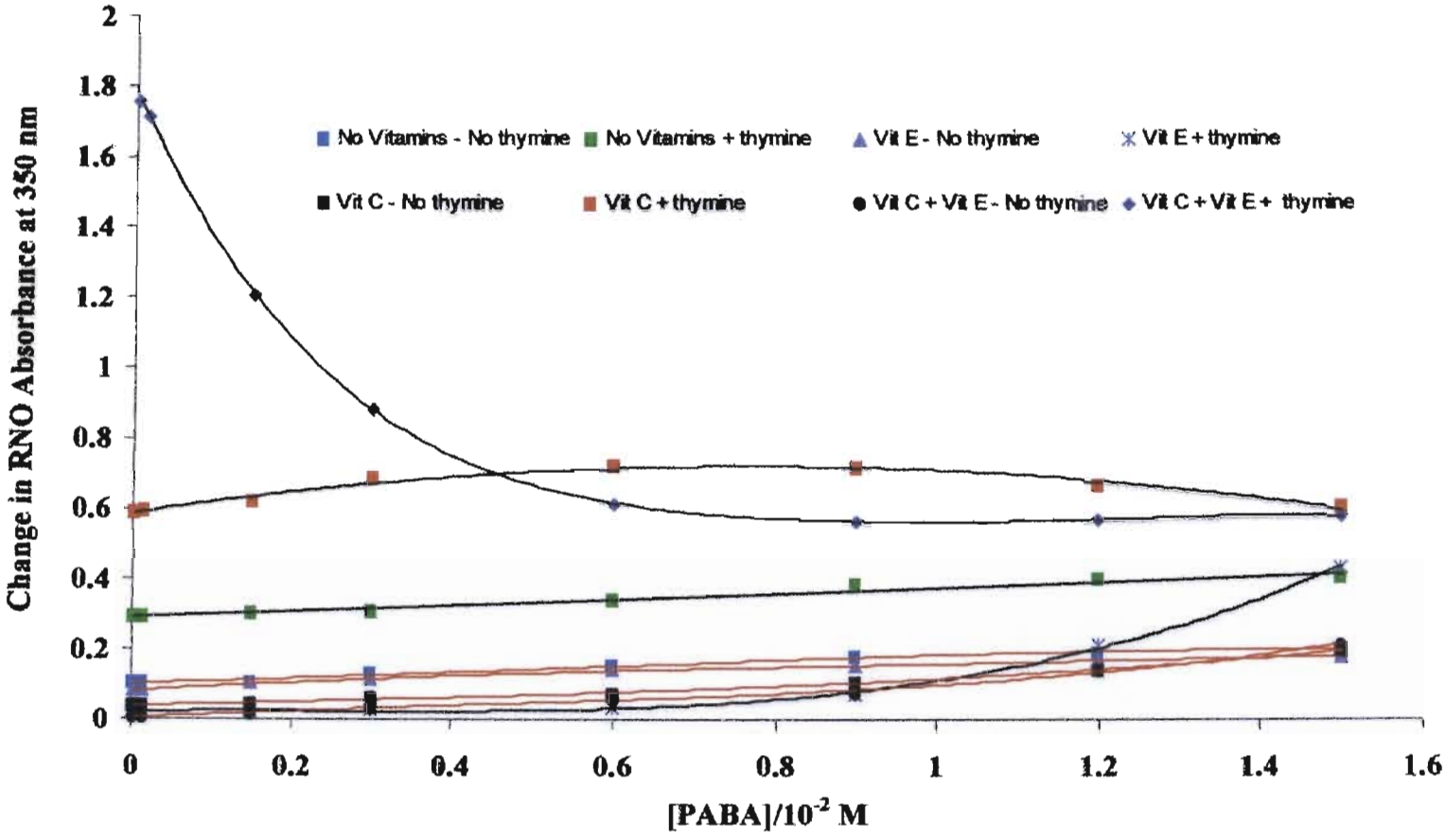

Figure 3.32: Comparison between the presence and absence of thymine and the effect of concentration of PABA on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for. The solution contained [RNO] = 4×10^{-5} M, [imidazole] = 8×10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1×10^{-2} M at pH 3. The presence of thymine is denoted by black lines and its absence by red lines.

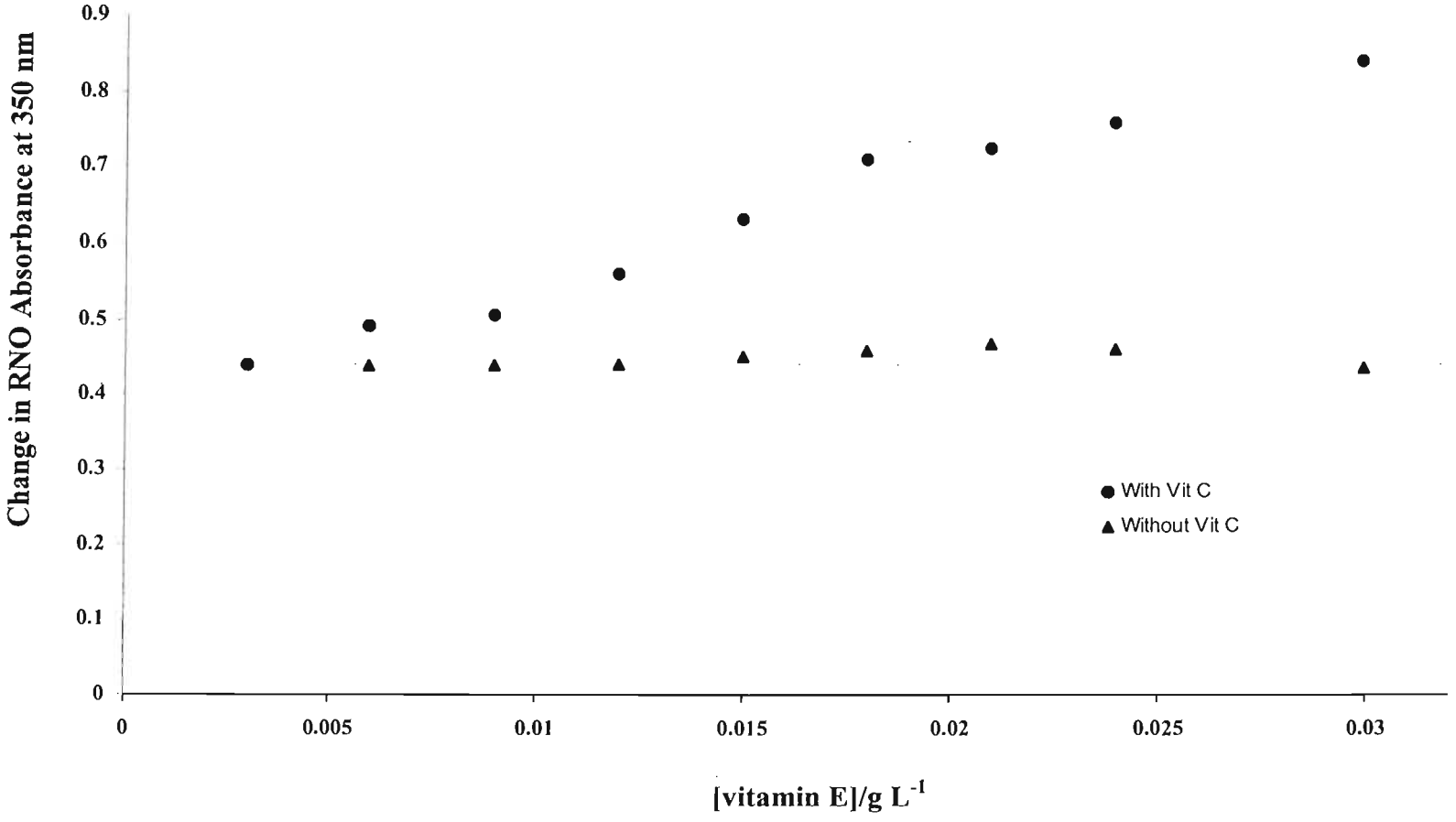

Figure 3.33: The effect of vitamin E concentration on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M at pH 3.0.

as a sink for singlet oxygen. Therefore, a plausible postulate for this observation is that the prooxidant property of vitamin E enhances this property of thymine. Addition of a constant amount of vitamin C increases the total amount of singlet oxygen formed. This formation increases monotonically with increase in vitamin E concentration. Firstly, vitamin E is known to act as a prooxidant at high concentrations. Secondly, it is known that vitamin C can be consumed in the presence of vitamin E for the regeneration of the latter. Therefore, as the concentration of vitamin E increases, that of vitamin C progressively decreases and hence, less of the vitamin is available for the quenching of singlet oxygen. These two factors, and the fact that thymine can also photosensitise the formation of singlet oxygen, could account for the increase in the total amount of singlet oxygen formed on addition of vitamin C.

Figure 3.34 combines the results from Figures 3.27 and 3.33. It compares the effect of adding thymine on the two sets of experiments. Without exception, all the graphs representing the presence of thymine are above those representing the absence of thymine. This implies that thymine has the effect of increasing the formation of singlet oxygen as the concentration of vitamin E increases irrespective of the presence or absence of vitamin C. This is not surprising given the fact that thymine can photosensitise the formation of singlet oxygen. Another factor that could be contributing to the enhancement of the formation of singlet oxygen besides thymine is the simultaneous presence of PABA and thymine. The two can produce triplet PABA and triplet thymine states that eventually result in singlet oxygen formation.

3.3.4 EFFECT OF VITAMIN C CONCENTRATION

Figure 3.35 shows graphs for the change in RNO absorbance measured at 350 nm against the concentration of vitamin C for a constant irradiation time of fifteen minutes. The following concentrations were constant: [RNO] = 4×10^{-5} M, [imidazole] = 8×10^{-3} M, [PABA] = 1×10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [thymine] = 1×10^{-2} M.

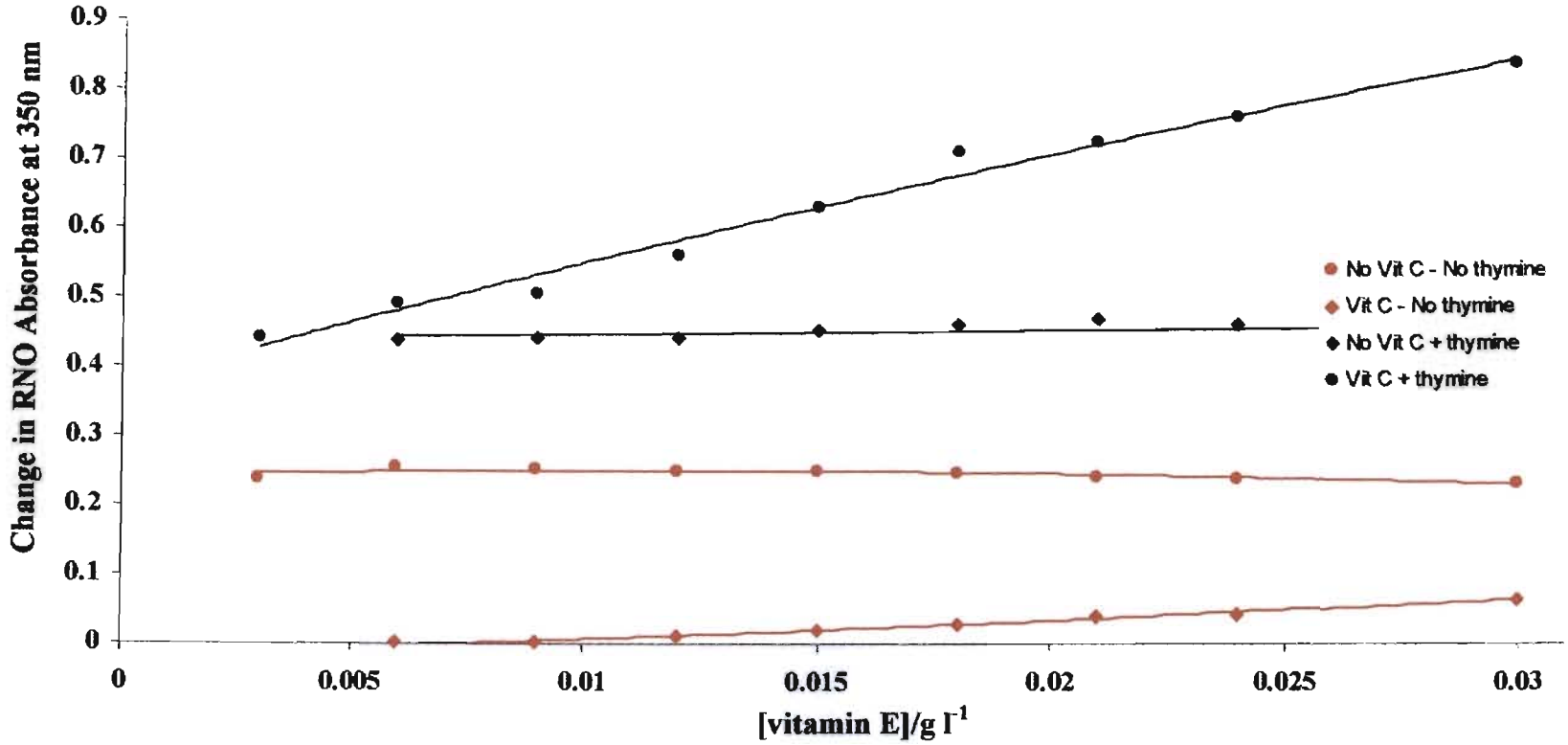

Figure 3.34: Comparison between the presence and absence of thymine and the effect of vitamin E concentration on singlet oxygen formation as determined by the change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M and [vitamin C] = 0.044 g L^{-1} and [thymine] = 1 x 10^{-2} M at pH 3.0. The presence of thymine is denoted by black lines and it absence by red lines.

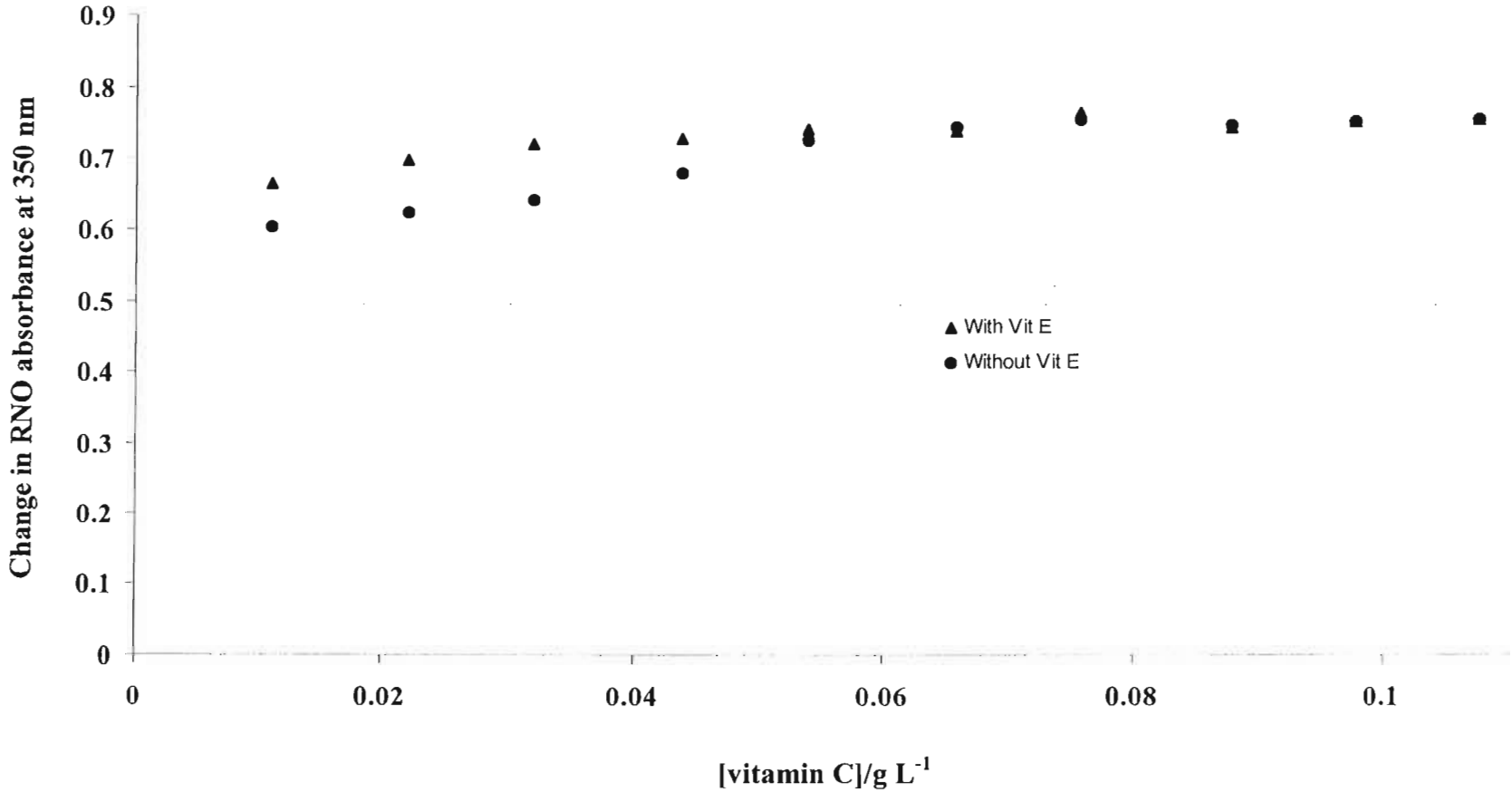

Figure 3.35: The effect of vitamin C concentration on singlet oxygen formation as determined by change in absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [thymine] = 1 x 10^{-2} M at pH 3.0.

Both graphs show an increase in the formation of singlet oxygen with an increase in the concentration of vitamin C. Both are in conflict with the postulate that at high concentrations vitamin C shows antioxidant properties and at low concentration prooxidant properties **[193]**. Nevertheless, it agrees with the rule of thumb that antioxidants properties diminish at high concentrations and instead prooxidant property takes over **[84]**. In both cases the increase stops at about 0.08 g L^{-1} of vitamin C. Thereafter, there is no observable change in the formation of singlet oxygen as the concentration of vitamin C is increased. For concentrations below 0.08 g L^{-1}, vitamin E has the effect of increasing the total amount of singlet oxygen formed and thereafter there is no effect, i.e. the change in absorbance is the same. The increase in formation of the total amount of singlet oxygen in the presence of both vitamins C and E could be due to the combined prooxidant activity, i.e. they are showing antagonism. It is possible that vitamin C is being consumed to regenerate vitamin E at low concentrations of vitamin C and that is why the formation of singlet oxygen is high in the presence of both vitamins. As the concentration of vitamin C increases, there is more than enough vitamin C for regeneration purposes and the total amount of singlet oxygen formed is no longer higher in the presence of vitamin E. There is no plausible reason as to why both graphs should level off at a high concentration of vitamin C other than that the optimal concentration has been reached at which antioxidant and prooxidant activities are balanced.

Figure 3.36 combines the results from Figures 3.28 and 3.35. It compares the effect of adding thymine on the two sets experiments. It is evident that the presence of thymine increases the formation of singlet oxygen irrespective of the presence of vitamin E as the concentration of vitamin C is increased. These observations could be due to the photosensitizing property of thymine as explained in the previous sections.

In conclusion, both antioxidant and prooxidant activities of the two vitamins can be exhibited with respect to singlet oxygen formation. Vitamins C and E can quench singlet oxygen that has been formed by PABA-photosensitisation. They can do this either individually or synergistically. For variable

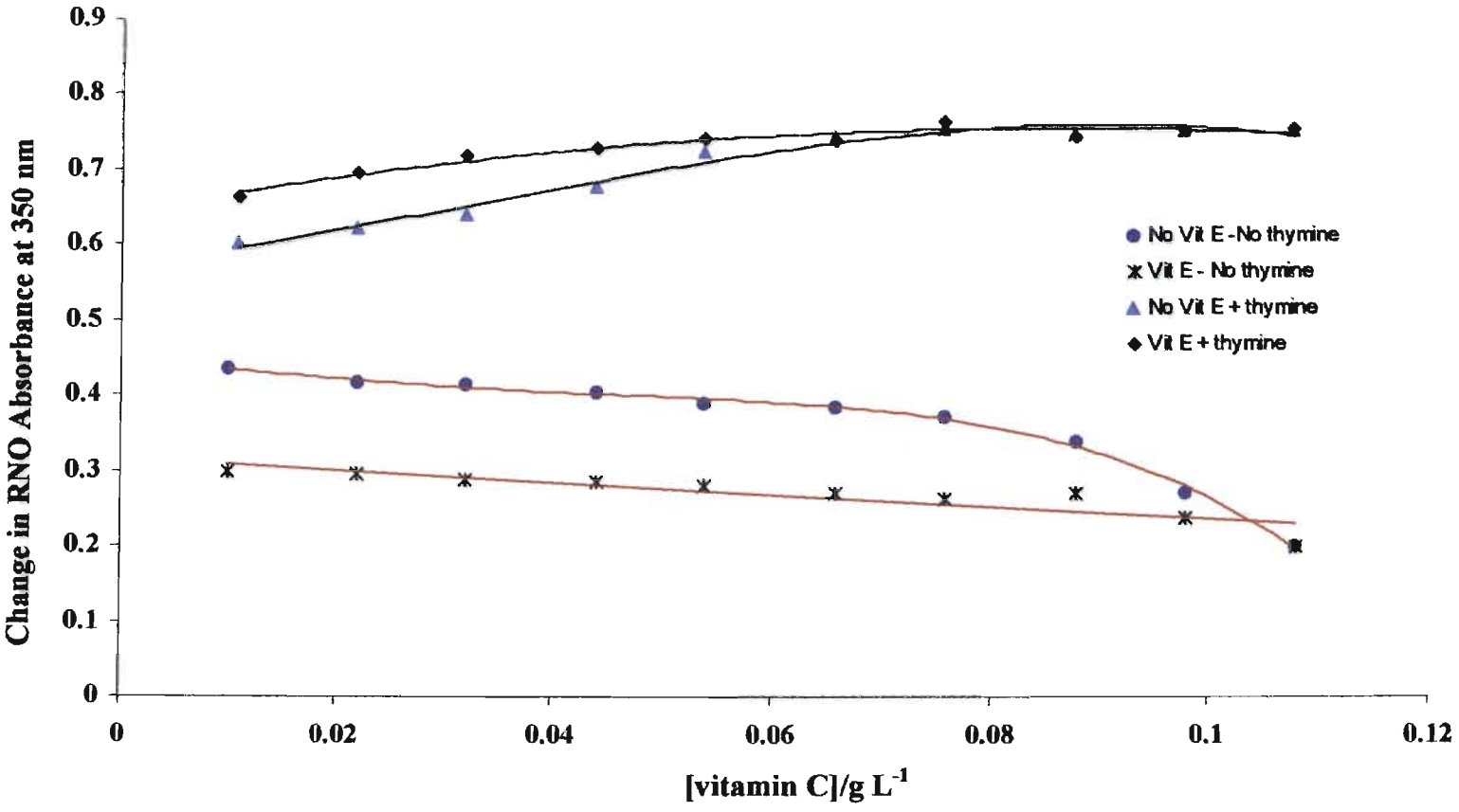

Figure 3.36: Comparison between the presence and absence of thymine and the effect of vitamin C concentration and thymine on singlet oxygen formation as determined by absorbance of RNO at 350 nm irradiated for 15 minutes. The solution contained [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [thymine] = 1 x 10^{-2} M at pH 3.0. The presence of thymine is denoted by black lines and its absence by red lines.

concentrations of the vitamins, synergism appears to be dominant but it depends on the individual amounts of the vitamins and PABA. In the presence of increasing concentrations of thymine, synergism is lost. This means that the vitamins can act as prooxidants with respect to singlet oxygen. In the presence or absence of the two antioxidants, the presence of thymine has the effect of increasing the total amount of singlet oxygen formed. The only observed exception to this is when thymine is present with vitamin E alone. With exception of PABA, generally in the presence of thymine, an increase in the variables investigated results in a monotonic increase in the formation of singlet oxygen. These observations strongly suggest that thymine is a photosensitiser of singlet oxygen and that even the two antioxidants are unable to counter its effect. The photosensitising ability of thymine is greatly reduced in the presence of triplet PABA at low PABA concentrations in the presence of both vitamins.

CHAPTER 4

CONCLUSIONS

Vitamin E and vitamin C are both antioxidants found in sunscreen formulations and are used for photoprotection against UV radiation. The photoprotection is due to both their scavenging and absorptive properties. PABA used until recently in sunscreens as an active ingredient has been shown to photosensitise the formation of thymine dimers. It has also been shown to be capable of photosensitizing the formation of singlet oxygen. Thymine dimer is a precursor for skin cancer where singlet oxygen has been implicated in many cases of oxidative stress, skin cancer, aging and cataract. The use of PABA as an active ingredient in sunscreens has declined primarily due to cases of photoallergy of PABA. The effect of introducing vitamin E and vitamin C individually or together on the PABA-photosensitized dimerisation of thymine was studied. This chapter draws conclusions from the data obtained by first looking at the specific sets of observations and secondly conclusions based on observations that cut across all the sets. The effect of both vitamin E and vitamin C on thymine dimer formation varied depending on their individual concentrations, their relative concentrations, whether they were present together or individually, the irradiation times and the concentrations of thymine and PABA. In some cases where the two antioxidants were not introduced the trend of the results agreed with findings of earlier workers in our research group **[74]**.

In the absence of any of the antioxidants, thymine dimer yield increases with an increase in irradiation time. This is due to an increase in the number of photons absorbed at longer irradiation hours. Introducing vitamin E increases the total dimer yield for short irradiation times but as irradiation time is increased, the total yield is lowered. This indicates a change in the activity of the vitamin with respect to time. Introduction of vitamin C alone decreases the total dimer yield significantly. The presence of vitamin E and vitamin C together had the lowest dimer yield in this set. In this set of irradiations, the most effective was a combination of the two vitamins, followed by vitamin C and lastly vitamin E.

There are reports in the literature alluding to the ability of vitamin E in reducing thymine dimer formation and also a combination of vitamins C and E **[86, 170]**. There are no reports in the literature alluding to the same effect with regard to vitamin C individually. Regarding a combination of the two vitamins, our findings agree with other researchers' findings **[86, 170]**. The presence of vitamin C enhanced the activity of vitamin E. For this set of irradiation, there is synergism between the vitamins. Generally, the relative dimer yield is in the order *cis-syn* < *cis-anti* < *trans-anti* < *trans-syn* but the absence or presence of the vitamins does not affect the relative yield.

In the absence of antioxidants, an increase in PABA concentration at constant thymine concentration and irradiation time generally decreases thymine dimer yield. This is not surprising since an increase in PABA concentration results in an increase in self-quenching of triplet PABA. Introducing vitamin E alone decreases the total thymine dimer yield. This observation can be attributed to the maximum antioxidant properties of vitamin E since the short irradiation time makes it less susceptible to undergo auto-oxidation. Introducing vitamin C alone increases dimer yield. It is still not clear how vitamin C chemically interacts with thymine to lower or increase the formation of thymine dimer. Nevertheless, DNA damage by vitamin C has been documented **[141, 190]**. The references quoted do not refer to thymine dimer formation explicitly as the damage caused by vitamin C on DNA. Our results are based on thymine dimer, therefore, there is strong indication that it can increase thymine dimer yield. This observation does not agree with the one in the previous section but the reasons could be due to the concentrations of the reagents and irradiation time. Introduction of vitamin E and vitamin C together increases dimer yield. As explained in Chapter 1, the presence of a photosensitiser can reduce the activity of both vitamins **[177]**. Here, the effect of an increase in the concentration of PABA could be overshadowing the synergism that we expect in such a situation. There have been cases reported where combined action of the two antioxidants does not produce the desired results of synergism **[264]**. With regard to relative dimer yield, the lowest was still *cis-syn* dimer while the highest was *trans-anti* dimer.

Thymine dimer yield increases with an increase in thymine concentration for constant irradiation time and constant concentration of PABA. This is as a result of an increase in the number of excited triplets of thymine which is necessary for the dimerisation. Addition of vitamin E decreases the total dimer yield substantially. Vitamin E is known to inhibit formation of cyclobutane pyrimidine dimers and specifically thymine dimer **[154]**. The exact mechanism by which it does so is not yet known but it is thought that both its antioxidant and absorptive properties play a role with the former likely to play the main role. Addition of vitamin C alone did not show any particular trend in the yield of thymine dimer. This could be due to both its pro- and anti-activity taking place concurrently. Introducing the two antioxidants together increases the total thymine dimer yield in this set of experiments. The most probable reason could that, with increase in the thymine concentration, more triplets of thymine are formed and overshadow the combined antioxidant properties of the two vitamins. Another possibility could be the increase in interaction between the two antioxidants in the presence of thymine to promote pro-oxidant activity of the two vitamins **[177]**. With regard to relative dimer yield, the yield was *cis-syn* < *trans-anti* < *cis-anti* < *trans-syn*.

For changing concentration of vitamin E, constant irradiation time and constant concentrations of thymine and PABA, there is a slight increase in thymine dimer yield as the concentration of vitamin E is increased. This is not surprising since vitamin E is known to lose its antioxidant activity at high concentration. Antioxidants are known to be pro-oxidant at high concentration. Maintaining the same conditions but introducing vitamin C lowers the total thymine dimer yield substantially. The amount formed in this case is almost constant except at the end (high vitamin E concentration) when the total yield rises. The plausible explanation is that synergism is playing a role but at very high vitamin E concentration its prooxidant properties overshadow the effect of synergism. The relative dimer yield was *cis-syn* < *cis-anti* < *trans-anti* < *trans-syn*.

For changing concentration of vitamin C, constant irradiation time and constant concentrations of thymine and PABA, there is an increase in total dimer yield

then a decrease. This might represent a change in the activity of vitamin C. There are conflicting reports regarding the activity of vitamin C relative to its concentration. Some research findings have reported that it is a prooxidant at low concentration and an antioxidant at high concentrations **[193]**, whereas some literature reports indicate that generally, antioxidants show prooxidant property at high concentration **[84]**. Therefore, our result i.e. an increase in thymine dimer yield in the presence of vitamin C agrees with the latter suggestion. It is very difficult to determine the reason why the yield falls after that. Addition of vitamin E substantially decreases dimer yield. This could be due to synergism between the two vitamins. *Cis-syn* dimer had the lowest yield with no particular trend for the other dimers.

From the above discussion both vitamin E and vitamin C can increase or decrease the total thymine dimer yield that results from PABA-photosensitisation. The increase or decrease depends on a number of factors. These factors are irradiation time, the composition and relative concentrations of the four reactants in the mixture. Both synergism and combined prooxidant (antagonism) activities of the two vitamins are exhibited with regard to thymine dimer formation. Again this depends on the four factors just mentioned. One fact is clear and that is, vitamin E does not show the ability to lower dimer formation at high concentration. PABA can therefore be considered "safe" in sunscreens if used together with the vitamins so long as the optimum conditions are determined and used.

Comparison and conclusions were based on total dimer yield and not on the yield of specific dimer isomers. The relative yield of the four dimer isomers differed from one set to another. There was no particular trend in the formation of isomers of the thymine dimer. Nevertheless, the yield for *cis-syn* dimer was the lowest in all the cases observed. *Cis-syn* thymine dimer is the one mostly associated with skin cancers because it is the only stereoisomer that forms in DNA. Two dimers, *cis-anti* and *trans-syn*, were always formed and could be detected. *Trans-anti* was either not formed or was formed but the yield was below the detection limit or could not be resolved from thymine. Since there

were no standards for *cis-anti* and *trans-syn* dimers, for these two cases the order was always assumed to be *cis-anti* followed by *trans-syn*.

The total dimer yield was of the order 10^{-6} M, or even less. From irradiation of the blanks, it is evident that irradiation of thymine in the absence of a photosensitiser can also form dimers. But as much as some dimers are formed, the yield is extremely low and long irradiation hours are required to produce the dimers. For investigations involving irradiation of a combination of two or more reagents (thymine, PABA, vitamin E and vitamin C), besides the dimers, many other photoproducts were formed on irradiation. This could be due to the reagents interacting with one another.

Separation, isolation and identification of all these compounds was not an easy task. Different sets of experiments produced different photoproducts. Further work needs to be done for the purpose of identifying these photoproducts. For 4% (v/v) methanol in the mobile phase, the order of elution of the dimers agreed with earlier findings by Cadet *et al.* **[212]**. For methanol content lower than 4% the order of elution did not agree with the one given by Cadet *el al.* α-Tocopherol acetate can reduce the PABA-photosensitised formation of thymine dimer and thus can be photoprotectant.

α-Tocopherol acetate absorbs relatively less strongly compared to α-tocopherol. Our investigations were performed in aqueous media and the most suitable substrate was the water-soluble acetate. This explains why the acetate could not lower the dimer yield as expected in certain cases. Vitamin E could not be traced in the photoproducts implying that either all of it was consumed or whatever remained was destroyed by UV radiation to give photoproducts that lost conjugation and hence could not be detected.

PABA is also known to photosensitise the formation of singlet oxygen. Vitamins C and E are antioxidants known to fight cancer and skin aging problems caused by free radical and reactive oxygen species like singlet oxygen. The effect of introducing vitamins E and C either individually or together on the PABA-photosensitized formation of singlet oxygen in the

presence or absence of thymine was studied. Solutions were prepared at pH 3.0 and irradiated with radiation of wavelength greater than 300 nm. The parameters investigated were the same as those in the photosensitised formation of thymine dimer by PABA. These were irradiation times and the concentrations of the antioxidants, thymine and PABA. The change in absorbance of RNO at 350 nm was measured and the amount of singlet oxygen formed inferred from these measurements. Comparisons were based mainly on the presence or absence of the antioxidants and thymine. Generally, the results from singlet oxygen studies showed more consistency than those of thymine dimer.

Singlet oxygen formation increased with an increase in irradiation time for all the sets of experiments. In these sets, both vitamins C and E and their combination reduced the total amount of singlet oxygen formed. The formation of singlet oxygen also increased with an increase in the concentration of PABA. This time again, the presence of both vitamins whether individually or together reduced the total amount of singlet oxygen formed. For the change in concentration of thymine, the formation of singlet oxygen also increased with increase in thymine concentration for all the sets of irradiations studied except for that in which the two vitamins were present together. In this set the formation decreased with increase in thymine concentration. Individually, both vitamins C and E reduced the total amount of singlet oxygen formed as the concentration of thymine increased but their combination did not reduce the total amount of singlet oxygen formed, instead it increased. For the individual vitamins, while increasing the concentration of vitamin E, the amount of singlet oxygen formed remained constant but it did increase with increase in concentration of vitamin C. The total amount of singlet oxygen formed decreased by introducing the second vitamin in both cases. We can conclude that generally, the vitamins exhibit antioxidant properties in terms of singlet oxygen formation individually or when together except in the presence of an increasing concentration of thymine where synergism between the two is lost. There is no reasonable explanation as to why this property of the vitamins is lost in the presence of thymine. A plausible reason could be that the photosensitising properties of thymine are greater than the effect of synergism.

The order of antioxidant activity for increase in irradiation time and PABA concentration: was vitamin E followed by vitamin C and lastly a combination of both as being the most effective in reducing formation of singlet oxygen. This order was reversed at the highest concentration of PABA.

A constant amount of thymine was introduced in the sets of irradiations described in the previous section. For all these sets of experiments, addition of thymine increased the total amount of singlet oxygen formed. There is only one exception to this, when the concentration of PABA was varied and vitamin E added. In this set, thymine had the effect of reducing the total amount of singlet oxygen formed. However, the reduction in amount of singlet oxygen formed was small that it was likely an experimental error. The order of quenching remained the same for an increase in irradiation time and an increase in PABA concentration in the presence of thymine. That is, vitamin C followed by a combination of both and lastly vitamin E, as the most effective in reducing the amount PABA-photosensitised singlet oxygen formation.

It is evident from the singlet oxygen studies that both antioxidants can quench PABA-photosensitised singlet oxygen. This happens whether they are acting individually or together with only one exception. Vitamin E was the most effective quencher of singlet oxygen in all the cases studied. Regarding the ability of vitamin C and a combination of both, the order depended on the presence or absence of thymine.

In conclusion, vitamins C and E generally can reduce the total amount of thymine dimer formed by PABA-photosensitisation. This happens either individually or together depending on the total irradiation time and the individual concentrations of the other reagents. The amount of *cis-syn* thymine dimer formed was the least relative to the other isomers. Both vitamins can also quench singlet oxygen formed by PABA-photosensitisation whether individually or together. The presence of thymine increases the formation of singlet oxygen despite the presence of the vitamins. The reaction mechanism

below is a postulate that could explain the action of thymine regarding singlet oxygen formation.

$$^1P + h\nu \rightarrow {}^1P^* \rightarrow {}^3P^*$$
$$^3P^* + T \rightarrow {}^3T^* + {}^1P$$
$$^3T^* + {}^3O_2 \rightarrow {}^1O_2^*$$

Therefore, the vitamins can be used in sunscreen to ameliorate the deleterious effect of UV radiation regarding thymine dimer and singlet oxygen but with limitation. The limitation is the total number of hours a subject is exposed in the sun and the concentration of the vitamins vis-à-vis other constituents.

As a basis for future work, we recommend investigation into the proposed mechanism of quenching of triplet thymine by vitamins E and C. Therefore lifetimes of both triplet thymine and triplet PABA in the presence of the vitamins need investigation. The mechanism for the formation of singlet oxygen by thymine-photosensitisation in the presence of the vitamins also needs to be investigated. In addition, identification of the unknown photoproducts that were seen in the chromatograms of the PABA-photosensitised formation of thymine dimers in the presence of the vitamins could throw some light on the activities of the vitamins in the presence thymine and PABA.

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APPENDIX A

MATERIALS

A1 CHEMICALS FOR HPLC

Chemical	Grade (% purity)	Manufacturer
Acetonitrile	HPLC (99.8%)	Riedel-de-Haën
Methanol	HPLC (99.9%)	BDH
Propan-1-ol	HPLC (99.5%)	Lab-Scan
Tetrahydrofuran	HPLC (99%)	Riedel-de-Haën
Perchloric Acid	70%	SAARCHEM
Helium	High purity (99.999%)	Afrox
Uracil	AR (99%)	Aldrich
Millipore-Q^{50} water*		

A2 CHEMICALS FOR PHOTODIMERISATION AND SINGLET OXYGEN EXPERIMENTS

Chemical	Grade(% purity)	Manufacturer
Nitrogen	High purity	Afrox
Thymine	AR (99%)	Sigma
Para-aminobenzoic acid	AR (99%)	SAARCHEM
Alpha-tocopherol acetate (Dry vitamin E 50% Cold Water Soluble Formulation)	AR (99%)	Hoffman-La Roche
Ascorbic acid	AR (99%)	BDH chemicals
Hydrochloric Acid	32%	SAARCHEM
L-Histidine hydrochloride monohydrate	AR	Sigma
N, N-dimethyl-4-nitrosoaniline (RNO)	AR (97%)	Aldrich
Citric acid monohydrate	99%	Riedel-de-Haën

Imidazole	AR (99%)	Sigma
di-potassium hydrogen orthophosphate	98%	Hopkin and Williams
Dry ice		National Chemical Products

NB* Millipore-Q^{50} water refers to water that has been double distlilled through Millipore Milli-Q apparatus consisting of ion-exchange resins and organic removing resins.

APPENDIX B

EQUIPMENT

B1 Irradiation Equipment

Equipment	Manufacturer
HBO 500 W high pressure mercury lamp	Osram
Powerpack for HBO lamp	Schrieber
500 mL Photochemical reactor vessel with 200 W medium pressure mercury lamp and powerpack	Hanovia
J-221 longwave UV intensity meter	Blak-Ray
A 10 mm thick pyrex filter	

B2 Equipment for UV-spectroscopy

Equipment	Manufacturer
DMS 300 UV/Vis Spectrophotometer	Varian
Lambda 35 UV/Vis Spectrophotometer	Perkin-Elmer
Ultrospec IIE Single Beam Spectrophotometer	LKB
Quartz Cells, 10 mm pathlength	Pesca Analytical

B3 Equipment for HPLC analysis

Equipment	Manufacturer
600 multisolvent delivery system	Waters
U6K Variable Injector	Waters
Autosampler Series 2000	Perkin-Elmer
600 Photodiode Array Detector	Waters

996 Photodiode Array Detector	Waters
7010 Rheodyne Injector	Waters
Pentium II 600 MHz Personal Computer	De' Marc
APCCII Personal Computer	NEC
950C deskjet printer	Hewlett Packard
Guard-Pak μ-Bondapak C_{18} precolumn insert	Waters
HPLC Columns: Spherisorb 5ODS(2)	Phenomenex
Ultracarb 5ODS(20)	Phenomenex
Nucleosil 100 C_{18} 250 x 4.6 mm	Grom Analytik
Hamilton 100 μl Airtight Syringe	Hamilton Company, Nevada, USA

B4 Equipment for IR-spectroscopy

15.011 ton manual hydraulic press	Specac
Impact 400D spectrophotometer	Nicolet
7440 Colour Pro plotter	Hewlett Packard

APPENDIX C

DATA SETS FOR THYMINE PHOTODIMERISATION EXPERIMENTS

This section presents data obtained for the yield of PABA-photosensitised thymine dimers for changing irradiation time at constant concentrations of thymine, PABA, vitamins E and C. Data is also presented for a constant irradiation time of six hours and for varying concentration of one of the reagents. By "dimer" is meant the total dimer yield calculated as described in Section 2.7. The values in parenthesis represent the standard deviation for the thymine dimer yield obtained after three injections of the sample.

Table C1: Dimer yield as a function of irradiation time at pH 3.0, [thymine] = 1×10^{-2} M and [PABA] = 1×10^{-3} M.

Time/hours	[Dimer]/10^{-6} M	Time/hours	[Dimer]/10^{-6} M
1	0.840 (1.092)	11	75.707 (8.916)
2	0.941 (0.1291)	12	10.435
3	3.125 (2.854)	14	31.498
5	8.330 (0.6322)	15	23.450
6	7.604	17	67.422 (5.445)
7	9.161 (1.456)	18	39.670 (0.7412)
9	15.475 (4.345)	19	82.699 (3.422)

Table C2: Dimer yield as a function of irradiation time at pH 3.0, [thymine] = 1×10^{-2} M, [PABA] = 1×10^{-3} M and [vitamin E] = 0.018 g L^{-1}.

Time/hours	[Dimer]/10^{-6} M
5	7.583
11	42.174
13	31.250
15	50.419
17	23.637 (0.3585)
19	43.4311 (1.544)
21	41.013
24	19.056 (0.3501)

Table C3: Dimer yield as a function of irradiation time at pH 3.0, [thymine] = 1×10^{-2} M, [PABA] = 1×10^{-3} M and [vitamin C] = 0.044 g L^{-1}.

Time/hours	[Dimer]/10^{-6} M	Time/hours	[Dimer]/10^{-6} M
1	4.414	11	3.168
3	1.940	13	3.478
5	3.309	15	2.723
7	4.125	17	4.557
9	3.387 (2.808)	19	4.205

Table C4: Dimer yield as a function of irradiation time at pH 3.0, [thymine] = 1×10^{-2} M, [PABA] = 1×10^{-3} M, [Vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

Time/hours	[Dimer]/10^{-6} M	Time/hours	[Dimer]/10^{-6} M
1	10.341	11	3.578
3	0.226	13	6.519
5	1.813	15	3.988 (1.4247)
7	1.892	17	2.861
9	1.877	19	6.576

Table C5: Dimer yield as a function of PABA concentration at pH 3.0, and [thymine] = 1×10^{-2} M.

[PABA]/10^{-3} M	[Dimer]/10^{-6} M	PABA]/10^{-3} M	[Dimer]/10^{-6} M
0.8	2.683	10	2.033
2	1.957	12	1.32
4	2.095	15	1.362
8	1.5051	20	0.752

Table C6: Dimer yield as a function of PABA concentration at pH 3.0, [thymine] = 1×10^{-2} M and [vitamin E] = 0.018 g L^{-1}.

[PABA]/10^{-3} M	[Dimer]/10^{-6} M	PABA]/10^{-3} M	[Dimer]/10^{-6} M
0.8	1.239 (4.1282)	10	0.975 (0.4615)
1	3.333 (3.4850)	12	0.626 (0.7505)
2	1.3010 (0.6157)	15	0.922 (1.5035)
4	0.877 (0.4671)	20	0.649 (1.8128)
8	1.021 (0.0169)		

Table C7: Dimer yield as a function of PABA concentration at pH 3.0, [thymine] = 1 x 10^{-2} M and [vitamin C] = 0.044 g L^{-1}.

[PABA]/10^{-3} M	[Dimer]/10^{-6} M	PABA]/10^{-3} M	[Dimer]/10^{-6} M
0.8	4.250	10	6.855
1	4.531	12	2.505
2	2.491	15	1.520
4	5.757	20	1.256
8	3.256		

Table C8: Dimer yield as a function of PABA concentration at pH 3.0, [thymine] = 1 x 10^{-2} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

[PABA]/ 10^{-3} M	[Dimer]/10^{-6} M	[PABA]/ 10^{-3} M	[Dimer] /10^{-6} M
0.2	6.190	10	2.746
1	4.579	12	6.941 (0.9245)
2	1.616	14	8.323
4	2.759	15	2.310 (1.133)
8	6.284	20	3.122

Table C9: Dimer yield as a function of thymine concentration at pH 3.0, and [PABA] = 1 x 10^{-3} M.

[Thymine]/10^{-3} M	[Dimer]/10^{-6} M	[Thymine]/10^{-3} M	[Dimer]/10^{-6} M
1	1.574 (0.011)	6	2.052 (1.421)
2	1.942 (0.006)	7	1.887 (0.560)
3	1.530 (0.698)	8	3.275 (1.386)
4	1.510 (0.968)	9	2.171 (0.884)
5	2.399 (1.705)	10	2.660 (1.994)

Table C10: Dimer yield as a function of thymine concentration at pH 3.0, [PABA] = 1 x 10^{-3} M and [vitamin E] = 0.018 g L^{-1}.

[Thymine]/10^{-3} M	[Dimer]/10^{-6} M	[Thymine]/10^{-3} M	[Dimer]/10^{-6} M
1	1.390 (0.478)	6	1.123 (0.195)
2	0.912 (0.121)	7	1.484
3	0.788 (0.047)	8	0.990 (0.299)
4	0.540 (0.395)	9	1.538 (0.388)
5	0.916	10	3.005 (0.431)

Table C11: Dimer yield as a function of thymine concentration at pH 3.0, [PABA] = 1 x 10^{-3} M and [vitamin C] = 0.044 g L^{-1}.

[Thymine]/10^{-3} M	[Dimer]/10^{-6} M	[Thymine]/10^{-3} M	[Dimer]/10^{-6} M
1	0.799	6	1.290
2	0.954	7	5.664 (6.127)
3	15.552 (0.2775)	8	2.964
4	1.113	9	6.815 (7.515)
5	14.104(1.619)	10	0.974

Table C12: Dimer yield as a function of thymine concentration at pH 3.0 and [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g l^{-1} and [vitamin C] = 0.044 g L^{-1}.

[Thymine]/10^{-3} M	[Dimer]/10^{-6} M	[Thymine]/10^{-3} M	[Dimer]/10^{-6} M
1	3.742	6	1.3742
2	2.583	7	2.299
3	2.132	8	1.055
4	1.971 (0.0767)	9	2.864 (0.5113)
5	2.365	10	2.210

Table C13: Dimer yield as a function of vitamin E concentration at pH 3.0, [thymine] = 1 x 10^{-2} M and [PABA] = 1 x 10^{-3} M.

[vitamin E]/g L^{-1}	[Dimer]/10^{-6} M	[vitamin E]/g L^{-1}	[Dimer]/10^{-6} M
0.003	2.867	0.018	4.948
0.006	3.417	0.021	3.742 (0.607)
0.009	3.450	0.024	4.972 (1.525)
0.012	4.194	0.030	3.686 (0.470)
0.015	3.694 (0.953)		

Table C14: Dimer yield as a function of vitamin E concentration at pH 3.0, [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and [Vitamin C] = 0.044 g L^{-1}.

[vitamin E]/g L^{-1}	[Dimer]/10^{-6} M	[vitamin E]/g L^{-1}	[Dimer]/10^{-6} M
0.003	1.829	0.018	0.787
0.006	1.307	0.021	1.028 (3.000)
0.009	0.729 (3.520)	0.024	0.539 (1.119)
0.012	0.756	0.030	5.037
0.015	0.720		

Table C15: Dimer yield as a function of vitamin C concentration at pH 3.0, [thymine] = 1 x 10^{-2} M and [PABA] = 1 x 10^{-3} M.

[vitamin C]/ g L^{-1}	[Dimer]/10^{-6} M	[vitamin C]/ g L^{-1}	[Dimer]/10^{-6} M
0.010	5.526	0.066	18.09 (7.421)
0.022	6.916 (0.875)	0.076	10.20
0.032	27.35 (31.92)	0.088	11.07
0.044	13.89 (12.093)	0.098	12.19 (1.725)
0.054	29.67 (28.746)	0.108	5.13 (0.678)

Table C16: Dimer yield as a function of vitamin C concentration at [thymine] = 1 x 10^{-2} M, [PABA] = 1 x 10^{-3} M and vitamin A 0.018 g L^{-1}.

[vitamin C]/ g l^{-1}	[Dimer]/10^{-6} M	[vitaminC]/ g l^{-1}	[Dimer]/10^{-6} M
0.010	6.205	0.066	14.98
0.022	6.511	0.076	23.58
0.032	3.333	0.088	8.820
0.044	5.100	0.098	7.604
0.054	11.64	0.108	6.672

APPENDIX D

DATA SETS FOR SINGLET OXYGEN EXPERIMENTS

This section presents data obtained on the yield of singlet oxygen by PABA-photosensitisation for changing irradiation times at constant concentrations of PABA, RNO, imidazole, vitamins E and C. Data is also presented for a constant irradiation time of 15 minutes, constant concentration of RNO and imidazole, and for varying concentrations of PABA, thymine and vitamins E and C. The data was also collected in the presence and absence of thymine.

Table D1: Change in absorbance at 350 nm for an irradiated mixture of [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1} and [vitamin C] = 0.044 g L^{-1}.

Irradiation Time /minutes	Change in Absorbance			
	Without vitamins	With vitamin C	With vitamin E	With vitamins C and E
0	0	0	0	0
2	0.0664	0.0319	0.0504	0.0451
4	0.1180	0.0558	0.0802	0.0701
6	0.1739	0.0816	0.1164	0.0875
10	0.2488	0.1233	0.1367	0.0960
14	0.3148	0.2039	0.2027	0.0891
17	0.3654	0.2400	0.2335	0.0866
20	0.4099	0.2603	0.2481	0.0875

Table D2: Change in absorbance at 350 nm for an irradiated mixture of [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [vitamin E] = 0.18 g L^{-1}, [vitamin C] = 0.044 g L^{-1} and varying PABA concentration.

[PABA]/ 10^{-2} M	Change in Absorbance			
	Without vitamins	With vitamin C	With vitamin E	With vitamins C and E
1.5	0.2011	0.1940	0.1796	0.2125
1.2	0.1885	0.1417	0.1676	0.1379
0.9	0.1798	0.1052	0.1501	0.0763
0.6	0.1501	0.0714	0.1405	0.0580
0.3	0.1274	0.0616	0.1121	0.0320
0.15	0.1050	0.0435	0.1001	0.0180
0.015	0.1052	0.0378	0.0801	0.0047
0.003	0.1029	0.037	0.0822	0.0033

Table D3: Change in absorbance at 350 nm for an irradiated mixture of [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [vitamin C] = 0.018 g L^{-1} and varying thymine concentration.

[Thymine]/ 10^{-3} M	Change in Absorbance			
	Without vitamins	With vitamin C	With vitamin E	With vitamins C and E
1	0.5047	0.4281	0.5211	0.7331
2	0.4209	0.3318	0.3402	0.6743
3	0.4406	0.3350	0.3704	0.6403
4	0.4607	0.3404	0.4023	0.6204
5	0.4806	0.3601	0.4390	0.6043
6	0.4950	0.3590	0.4677	0.6097
7	0.5216	1.3004	0.4839	0.6058
8	0.5405	0.4083	0.5073	0.5816
9	0.5530	0.4250	0.5347	0.5829
10	0.5804	0.4650	0.5135	0.7164

Table D4: Change in absorbance at 350 nm for an irradiated mixtur
= 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M [PABA] = 1 x 1
[vitamin C] = 0.044 g L^{-1} and varying vitamin E concentration.

[vitamin E]/g L^{-1}	Change in Absorbance	
	Without vitamin C	With vitamin C
0.003	0.2401	-0.0114
0.006	0.2568	0.0026
0.009	0.2521	0.0032
0.012	0.2505	0.0102
0.015	0.2495	0.0181
0.018	0.2473	0.0280
0.021	0.2429	0.0394
0.024	0.2397	0.0423
0.030	0.2306	0.0652

Table D5: Change in absorbance at 350 nm for an irradiated mixture of [RNO]
= 4 x10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M,
[vitamin E] = 0.018 g L^{-1} and varying vitamin C concentration.

[vitamin C]/g L^{-1}	Change in Absorbance	
	Without vitamin E	With vitamin E
0.011	0.3309	0.2987
0.022	0.4192	0.2973
0.032	0.4149	0.2899
0.044	0.4056	0.2877
0.054	0.3902	0.2810
0.066	0.4050	0.3957
0.076	0.3722	0.2634
0.088	0.3387	0.2705
0.098	0.2716	0.2389
0.108	0.1999	0.2002

Table D6: Change in absorbance at 350 nm for an irradiated mixture = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10 [vitamin C] = 0.044 g L^{-1}, [vitamin E] = 0.018 g L^{-1} and [thymine] = 1 x 10^{-2} M for varying irradiation times.

Irradiation time/minutes	Change in Absorbance			
	Without vitamins	With vitamin C	With vitamin E	With vitamins C and E
0	0	0	0	0
2	0.0776	0.2503	0.0746	0.1923
4	0.1150	0.4386	0.1224	0.3193
6	0.1603	0.5659	0.1647	0.3796
10	0.2307	0.6162	0.2216	0.4219
14	0.2972	0.6232	0.2718	0.4196
17	0.3474	0.6292	0.2956	0.4143
20	0.3635	0.6325	0.3172	0.4185

Table D7: Change in absorbance at 350 nm for a 15-minute irradiated mixture of [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [vitamin E] = 0.18 g L^{-1}, [vitamin C] = 0.044 g L^{-1}, [thymine] = 1 x 10^{-2} M and varying PABA concentration.

[PABA] /10^{-2} M	Change in Absorbance			
	Without vitamins	With vitamin C	With vitamin E	With vitamins C and E
1.5	0.4035	0.6125	0.4341	0.5813
1.2	0.3985	0.6716	0.2090	0.5707
0.9	0.3852	0.7199	0.0725	0.5656
0.6	0.3417	0.7233	0.0315	0.6172
0.3	0.3039	0.6886	0.0287	0.8802
0.15	0.3028	0.6194	0.0215	1.2067
0.015	0.2940	0.5931	0.0232	1.7111
0.003	0.2925	0.5890	0.0229	1.7570

Table D8: Change in absorbance at 350 nm for an irradiated mixtu
= 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x
[vitamin C] = 0.044 g L^{-1}, [thymine] = 1 x 10^{-2} M and vary
vitamin E concentration.

[vitamin E]/g L^{-1}	Change in Absorbance	
	Without vitamin C	With vitamin C
0.003	0.4406	0.3425
0.006	0.4921	0.4399
0.009	0.5059	0.4412
0.012	0.5604	0.4426
0.015	0.6313	0.4521
0.018	0.7099	0.5036
0.021	0.7256	0.4693
0.024	0.7605	0.4624
0.030	0.8402	0.4380

Table D10: Change in absorbance at 350 nm for an irradiated mixture of [RNO] = 4 x 10^{-5} M, [imidazole] = 8 x 10^{-3} M, [PABA] = 1 x 10^{-3} M, [vitamin E] = 0.018 g L^{-1}, [thymine] = 1 x 10^{-2} M and varying vitamin C concentration.

[vitamin C]/g L^{-1}	Change in Absorbance	
	Without vitamin E	With vitamin E
0.010	0.6027	0.6642
0.022	0.6240	0.6973
0.032	0.6409	0.7211
0.044	0.6806	0.7297
0.054	0.7257	0.7432
0.066	0.7451	0.7400
0.076	0.7551	0.7661
0.088	0.7478	0.7453
0.098	0.7528	0.7526
0.108	0.7542	0.7550

APPENDIX E

POSTERS AND SEMINAR PRESENTED ON THIS WORK

This section gives details of conferences in which posters were presented and a seminar given during the course of this work.

E1 Ali M Salim and B S Martincigh, Effect of antioxidants on the *para*-aminobenzoic acid photosensitised reaction of thymine base, European Society for Photobiology Symposium on Photoprotection, Kraków, Poland, 19th to 22nd May 2001.

E2 Ali M Salim and B S Martincigh, The effect of vitamin E on the *para*-aminobenzoic acid photosensitised reaction of thymine base, 36th Convention of the South African Chemical Institute, Port Elizabeth, South Africa, 1st July to 5th July 2002.

E3 Ali M Salim, Photosensitised dimerisation of thymine in the presence of some antioxidants, Seminar presented in the School of Pure and Applied Chemistry, University of Natal, Durban, South Africa, 23rd May 2003.

E4 Ali M Salim and B S Martincigh, The effect of antioxidants on the *para*-paminobenzoic acid photosensitised reaction of thymine base, XXth International Union of Pure and Applied Chemistry Symposium on Photochemistry, Granada, Spain, 17th July to 22nd July 2004.

E5 Ali M Salim and B S Martincigh, The effect of antioxidants on *para*-aminobenzoic acid photosensitized formation of singlet oxygen, 11th Congress of The European Society for Photobiology, Aix-les-Bains, France, 3rd September to 8th September 2005.