EFFECT OF WHEY PROTEIN ISOLATE ON THE OXIDATIVE STABILITY OF VITAMIN A

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"THE ONE WHO CAN DISCIPLINE THEIR MIND CAN STEP OUT OF THIS ENDLESS CYCLE OF DESIRE AND AVERSIONS."

- GAUTAMA BUDDHA

ABSTRACT

The purpose of this study was to investigate the possible protective effect of whey protein isolate (WPI) on the oxidative stability of vitamin A in an aqueous phase. The first part of the study focused on the development of a reliable method to extract retinol from the samples for analysis. Direct solvent extraction and saponification were both tested, with saponification, which converts retinol acetate into retinol, chosen for the experimental work. Extracted retinol was quantified using reversed phase HPLC, to obtain the degradation trends of the samples that had been subjected to various conditions.

During the second part of the study, the degradation trends were obtained for the samples of retinol acetate in the presence and absence of WPI, when subjected to fluorescent light, pasteurisation, UHT treatment or storage at 5 or 40 °C. Samples exposed to fluorescent light at 4 °C showed exponential degradation of retinol acetate. Within 48 hours of light exposure, almost 60% of the retinol acetate had degraded regardless of the initial concentration. However, samples containing WPI retained slightly more retinol acetate at initial retinol acetate concentrations > 25 μg/ml.

The presence of WPI had a protective effect on retinol acetate during pasteurisation at 72 °C for 15 seconds. This protective effect appeared to be associated with the WPI concentration. However, there was no difference between samples with or without WPI after UHT treatment at 144 °C for 3-4 seconds, presumably due to the denaturation of the whey proteins.

The effect of WPI on retinol acetate during storage was minimal at 40 °C, where total degradation of retinol occurred within 48 hours of storage regardless of the initial retinol acetate concentration. In contrast, WPI showed a significant protective effect on retinol acetate at 5 °C, especially when the initial retinol acetate concentration was > 25mg/ml.

Overall, the presence of WPI and higher initial retinol acetate concentrations showed better stability than the control samples when exposed to light, stored at 5°C or during pasteurisation.

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CHAPTER 1

INTRODUCTION

If vitamin A is to be incorporated into a food or beverage then the vitamin must be sufficiently stable to ensure adequate potency in the product throughout the shelf-life. This is an issue due to the vitamin A losses that occur mainly through oxidation or geometric isomerisation. While oxidation results in total degradation of vitamin A, isomerisation reduces the vitamin A activity. The focus of this study was on stabilising vitamin A against the oxidation.

The main factors affecting vitamin A stability are light, storage and processing temperature. Ultraviolet (UV) rays can either result in photo-oxidation or photo-isomerisation. Thermal stresses triggered by elevated storage temperature and high processing temperature also result in the oxidation and isomerisation of vitamin A. Much research has focused on stabilising retinol in the presence of these conditions using physical barriers such as colour pigmented packaging, UV absorbers and wavelength transmitting filters (Sattar *et al.*, 1977; Fanelli *et al.*, 1985; Cladman *et al.*, 1998). Recently, physico-chemical approaches such as the use of emulsion systems, encapsulation and vitamin A carriers have also been reported (Zahar *et al.*, 1986; Halbout *et al.*, 1997; (Beaulieu *et al.*, 2002). It has also been suggested a carrier molecule that can bind to vitamin A and thus reduce its reactivity may be able to improve its stability.

Whey protein isolate (WPI) comprises β-Lg, α-La, BSA and immunoglobulins, with β-Lg representing approximately 50 % of the total protein. Preliminary studies on WPI have shown that retinol as well as retinol acetate bind to WPI in an aqueous environment (Schofield, 1999).

Vitamin A has been shown to bind to certain whey proteins. Retinol is known to bind to the most abundant whey protein, β-lactoglobulin (β-Lg) as well as to α -

lactalbumin (α-La) and bovine serum albumin (BSA). Bovine β-Lg consists of eight anti-parallel β-sheets that form a calyx, which is flanked on one side by an α-helix, forming a hydrophobic pocket (Dufour *et al.*, 1991). It has been shown that retinol binds in this hydrophobic central calyx (Kontopidis *et al.*, 2002). In addition, retinol is also known to bind at an external binding site that is located on the surface of the β-Lg molecule (Monaco *et al.*, 1987). Futterman *et al.* (1972) has reported retinol binds to BSA and forms water-soluble complexes. Therefore, whey proteins appear to have the potential to be used as carrier molecules to stabilise vitamin A in an aqueous environment. Most importantly, the potential use of β-Lg as a carrier molecule for lipophilic fragments, such as retinol, has been patented (Swaisgood *et al.*, 1998).

Vitamin A naturally present in foods is known to be associated with the lipid components. There are many fat-free or low fat food products currently available, which are usually also low in fat-soluble vitamins. Although many other vitamin A carrier emulsion systems have been reported in the literature (Zahar *et al.*, 1986), they contain fat components (such as butter, coconut and peanut oil) and thus affect the total fat composition of the product. The advantages in the use of WPI to stabilise vitamin A in low-fat food products include its ability to solubilise the vitamin without the addition of fat. However, if vitamin A is to be incorporated into a food or beverage product using WPI, it must be ascertained that the added vitamin A remains above a minimum level during the shelf –life of the product.

The purpose of this study was to gain a better understanding of any possible protective effect of WPI on vitamin A under a range of conditions. This involved finding appropriate methodologies for extracting and quantifying the added retinol acetate and the studying of degradation trends of the added retinol in the presence of the light, storage and processing temperatures.

CHAPTER 2

LITERATURE REVIEW

This literature review covers a range of topics from the biochemistry to the novel approaches of stabilising vitamin A. The nomenclature, stability, factors that affect the stability, modes of degradation and the analytical methods of vitamin A are the key topics addressed in the first half of the review. The second half of the review focuses on the methods of stabilising the vitamin including the suitability and the possibility of the use of whey proteins.

2.1 Vitamin A

The term, vitamin A, refers to a group of nutritionally active unsaturated hydrocarbons, known as retinoids. The structure of retinoids consists of a betaionone ring, an unsaturated hydrocarbon chain with adjoining reactive group that could be an alcohol, aldehyde, ester or carboxylic acid. Vitamin A exists in two principal forms in nature, the preformed vitamin A and provitamin A. Retinol ($C_{20}H_{30}O$) is the major preformed vitamin A compound, and is systematically named as 9,13-dimethyl-7 (1,1,5 trimethyl-6-cyclohexen-5-yl)-7,9,11,13-nonatetraen-15-ol (Figure 2.1). This form of vitamin A is mainly found in animal sources such as liver, whole milk, butter, cheese, fish and egg yoke. Retinol forms esters with long chain fatty acids. Retinyl palmitate ($C_{36}H_{60}O_2$) and retinyl acetate ($C_{22}C_{32}O_2$) are retinol esters that can be synthesised chemically and are commonly used for vitamin A fortification in the food industry. Provitamin forms of vitamin A are commonly known as carotenoids. These are mainly plant derivatives although they can be found in animal sources, which have derived the carotenoids from the plant materials in their diet. The most well known of the carotenoids is beta-carotene, which is abundant in fruits and vegetables that are rich in yellow, or orange colours (e.g.; carrots, papaya, pumpkin) and in leafy green vegetables (e.g. broccoli and spinach).

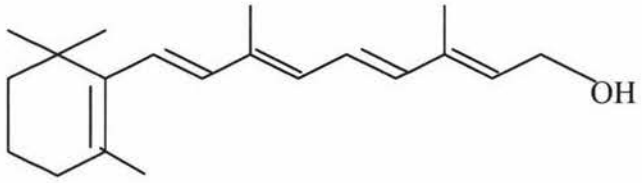

Figure 2.1 Chemical Structure of vitamin A, all-*trans*- retinol

Vitamin A is composed of three unique structural domains, a beta-ionone ring, a polyunsaturated C chain and a polar end group. The non-oxygenated beta-ionone ring is a structural characteristic that is essential for a compound to have vitamin A activity (Fennema, 1996). The double bonds in the carbon chain can result in structural isomerism of the parent compound, all-*trans*- retinol. The predominant isomers are 9-, 11- and 12-cis retinol. The polar group at the end of the isoprenoid side chain can be an alcohol, aldehyde, ester or a carboxylic acid. Vitamin A is fat-soluble and neither retinol nor its derivatives are soluble in water. However, vitamin A displays considerable solubility in alcohol, diethyl ether, petroleum ether, chloroform and acetone and various oils (Ball, 1998).

Since the dominant structural feature of retinol is the large number of conjugated double bonds, this means that the stability of retinol and its derivatives are affected by oxidising conditions (Woolard *et al.*, 2003). Retinoids are known to be susceptible to many environmental factors. Oxygen, light, processing/storage temperature, pH and the presence of fat are factors that greatly influence vitamin A stability (Herrero-Barbudo *et al.*, 2005). These environmental stresses can result in isomerization, oxidation and ultimately molecular cleavage leading to total destruction of the vitamin.

Vitamin A activity in food is expressed in international units (IU), where 1 IU = 0.3 μg. However, the bioavailability (the proportion of the quantity of vitamin A ingested that is absorbed and utilised by the body) is expressed in retinol equivalents (RE). 1 RE is defined as 1μg of all-*trans*-retinol (Bender, 2003), thus 1R.E = 3.33IU. The

factors that affect the stability of vitamin A, namely light, oxygen, temperature, etc., may either destroy the vitamin or reduce its biopotency. The biopotency of vitamin A, is an indication of its overall activity. For example, *cis* isomers, which may arise through exposure to light have reduced vitamin A activity compared to the all-*trans* isomers (Murphy *et al.*, 1988).

2.2 Methods of vitamin A extraction and analysis

Certain components in a food sample (such as lipid materials) exhibit similar solubility properties to fat soluble vitamins and can interfere with the separation and measurement of the vitamins. For example, naturally present vitamin A in foods is bound to lipoprotein complexes, and in milk, emulsifiers cause the fat and protein to interact strongly (Ball, 1988). Therefore, the first task in the analytical procedure is to disrupt the fat-protein interaction and extract the vitamin A from the food matrix. The two most common methods used are saponification and solvent extraction. The extracted vitamin can then be quantified using a variety of analytical techniques including spectrophotometry, fluorometric methods, colorimetry and chromatography. High performance liquid chromatography (HPLC) is the most widely used method (Ball, 1998).

2.2.1 Saponification

Saponification requires an alkali treatment, which releases the vitamin A from the food matrix and converts the ester form of retinol to the alcohol form. Saponification is an economical way of digesting large amount of materials and is an accepted method of extracting vitamin A from almost any type of food (Ball, 1988). This alkali treatment facilitates the hydrolysis of retinol esters to their respective alcohols, providing an estimate of the total retinol content.

2.2.2 Solvent extraction

Solvent extraction uses a series of washing steps to extract the lipid fraction of the food sample. The most commonly used solvents are hexane, acetone, isopropanol and chloroform. The solvent(s) to be used is dependent on the nature of the sample and the amount of lipid to be extracted. Ball (1988) states that "the more polar the solvent, more efficient is the extraction". However, this also results in the extraction

of more of the interfering materials such as fatty acids. "The solvent effectively breaks the lipoprotein bonds, while minimising oxidative destruction of the vitamin and lipids" (Ball, 1998). Solvent extraction allows the individual esters of vitamin A to be subsequently determined.

2.2.3 Ultraviolet spectroscopy

Spectrophotometry is best utilised as a means of detection in HPLC. It is based on the absorption of the UV or visible region of the spectrum by the vitamin molecules due to an electron transition within their structure. The absorbance value is proportional to the concentration of the irradiated compound. The radiation wavelength selected is the peak of the absorption spectrum (λ_{max}) for a purified vitamin A solution. For all-*trans*-retinol, this peak occurs at 325 nm. The main disadvantage of this method is the possible irrelevant absorption that could be caused by other substances that absorb light between 300 to 350 nm of the spectrum, leading to an overestimation of the vitamin A content (Ball, 1988). Extraction methods described in section 2.2.1 and 2.2.2 should reduce or eliminate such effects.

2.2.4 Fluorometric methods

Fluorescence spectrophotometry is based on the irradiation of a sample with UV light. Certain compounds will emit the radiation energy in the form of fluorescence. This fluorescence is then isolated by means of filters or by a monochromator with a desired excitation wavelength. The fluorescence emission is detected by a photomultiplier at right angles to the incident beam, thus the emitted radiation is detected against a relatively dark background (Ball, 1988). Retinol and retinyl esters fluoresce very strongly, upon excitation with long-wave UV light at 330 – 360 nm, and emit between 470 – 490 nm (Hubbard *et al.*, 1971). The fluorescence of retinol is a linear function of concentration. Different retinol isomers exhibit different degrees of fluorescence allowing the quantification of different retinol isomers. Certain structural features of a molecule are required for it to fluoresce, enabling this method to avoid the interference from other compounds such as sterols, and providing a higher specificity of detection. This means fluorometry is more sensitive than absorption spectrophotometry (Ball, 1988).

2.2.5 Colorimetry

The colorimetric method is based on a reaction between retinol and a group of acidic reagents known as Lewis acids (i.e. antimony trichloride, trifluoroacetic acid and trichlroacetic acid) resulting in the formation of a colourful complex. The absorbance of this complex at 616 - 620 nm is proportional to the concentration of vitamin A (Woolard *et al.*, 2003). This method is also known as the traditional Carr-Price method. This technique is non-specific so requires prior purification of the vitamin to eliminate interfering substances (Ball, 1988) and it is not capable of distinguishing between the various isomers. The other disadvantage of this method is that the colour is transient, and difficult to control, so the absorbance must be read within a few seconds (Ball, 1988).

2.2.6 Chromatography

It is widely accepted that a reliable estimate of vitamin A content of foods may be best achieved through using chromatography to separately quantify all active forms of retinol. Most types of chromatography have been used to quantify retinol. Thin-layer chromatography (TLC) and low-pressure liquid chromatography (LPLC) lack the resolving power for reliable quantification, and gas liquid chromatography may cause the degradation of the retinol at the elevated temperatures used by the technique. The use of HPLC has dominated the literature on vitamin A quantification, due to its accuracy, precision, speed and ease of use (Ball, 1988). HPLC allows the rapid and non-destructive quantification of the different vitamin A isomers at room temperature.

HPLC consists of a solvent system known as the mobile phase, a high-pressure pump, a column packed with extremely small particles (the stationary phase) and an absorption detector. Separation of different forms of retinol is achieved by applying a high pressure to force the mobile phase through the column. Specific interactions between the vitamin molecules, and the stationary and mobile phases result in different residence times within the column for different chemical structures, enabling their separation (Ball, 1988). The detection in HPLC relies mainly on the UV absorption or fluorescence spectral characteristics of the vitamin, which provide higher sensitivity and separation capacity. However, fluorescence detectors provide lower detection limits than the absorbance detectors (Ball, 1988). Although

electrochemical and mass spectrometric detections techniques have been reported, applications of these methods appear to be limited.

The two common modes of HPLC are known as normal-phase and reversed-phase HPLC. Normal-phase HPLC uses a non-polar mobile phase (i.e. hexane) and a polar stationary phase (i.e. silica). This provides an efficient mechanism for isomer separation. Panfili *et al.* (1998) followed the isomerization of naturally present vitamin A (all-*trans*-) in milk, to 13-*cis*-retinol, using normal phase HPLC. Reversed-phase HPLC uses a polar mobile phase (i.e. methanol) or simple binary combination of solvents (i.e. methanol and water) and a non-polar stationary phase (i.e. hydrocarbon). Reversed-phase separations are usually confined to C_{18} bonded-phase columns and are often used for total vitamin A quantification (Woolard *et al.*, 2003).

2.3 Modes of vitamin A degradation

The stability of vitamin A is affected by both extrinsic (light, oxygen, heat) and intrinsic (pH, presence of fat) factors (as discussed in section 2.4). These may trigger reactions involving the unsaturated isoprenoid side chain, which result in oxidation and geometric isomerization (Ball, 1998). Oxidation occurs mainly as auto-oxidation and photosensitised or light induced oxidation. Light and high temperatures usually result in degradation of the vitamin by isomerization, although lower pH values are also known to stimulate isomerization. Vitamin A degradation may also occur through de-esterification, which is mainly triggered by pH.

2.3.1 Auto-oxidation

Auto-oxidation is a self-catalytic mechanism that occurs *via* free radicals. The degradation of vitamin A displays typical characteristics of the autooxidation of unsaturated lipids (Fennema, 1996). The conjugated double bonds in retinoids provide an electron dense region, attracting electron deficient species such as free radicals. Radicals consist of a minimum of one unpaired electron and are highly reactive. Radical reactions comprise of three distinguishable steps known as initiation, propagation and termination. Initiation is a process where an initiator (I) produces radicals (R•) from non-radical reactants such as unsaturated lipids (RH).

$$I + RH \longrightarrow IH + R\bullet$$

Molecular oxygen in its excited singlet state can act as an initiator (Min *et al.*, 2002). Initiation is a slow process that is why auto-oxidation reactions typically show a "lag phase".

The newly formed radicals attack electron-dense bonds (often double bonds) and create more radicals (R•). These free radicals continue the "propagation" phase of autoxidation where the following sequence of reactions is repeated.

$$ROO\bullet + RH \longrightarrow ROOH + R\bullet$$

$$R\bullet + O_2 \longrightarrow ROO\bullet$$

Propagation occurs via the extraction of hydrogen atoms from the double bonds of the side chain of the vitamin A, which results in hydroperoxides (ROOH) and new free radicals (R•). Another path of the propagation reaction is the addition of oxygen across double bonds, creating peroxy radicals (ROO•). It is important to note that radical scavenging effect of antioxidants may break this chain reaction by inhibiting either the initiation or propagation phase.

Once the concentration of radicals increases or the concentration of reactants decreases, the radicals may react with each other. This is known as the "termination" phase, and produces non-radical products.

$$R\bullet + R\bullet \longrightarrow R\text{-}R$$

$$ROO\bullet + ROO\bullet \longrightarrow ROOR + O_2$$

2.3.1.1 Photochemical oxidation

Oxidative degradation of the vitamin A is generally parallel to the oxidative degradation of unsaturated lipids (Fennema, 1996). Photochemical oxidation occurs

via a photosensitiser, which absorbs the ultraviolet (UV) or visible radiation energy and becomes an unstable, excited, singlet state molecule known as singlet sensitiser (Min *et al.*, 2002). Many food components can act as photosensitisers, including colour pigments such as chlorophyll, synthetic colorants, vitamins such as riboflavin and proteins such as myoglobins (Foote *et al.*, 1968; Lledias *et al.*, 2000). The singlet sensitiser may directly catalyse the decomposition of the unsaturated side chain of vitamin A, or it may also undergo an "intersystem crossing" from the excited singlet state to an excited triplet state molecule, which triggers two photo-oxidation pathways. Pathway I involves the triplet state oxygen molecule reacting directly with a compound (RH) to produce free radicals, and leads to autooxidation. Pathway II involves in the formation of non-radical singlet oxygen from triplet oxygen in the presence of light. This occurs where the excited triplet sensitiser reacts with a triplet oxygen molecule creating a singlet oxygen molecule and a singlet sensitiser. Overall, pathway II provides more singlet sensitisers and singlet oxygen molecules to continue the photo-oxidation procedure. The rate of singlet oxidation is much greater than the oxidation caused by atmospheric triplet oxygen due to the low activation energy (Min *et al.*, 2002).

2.3.2 Geometric isomerization

Geometric isomerization changes the form of the vitamin A, rather than causing its total destruction. The double bonds present at the 9, 11, and 13 positions of the unsaturated side chain can undergo *cis-trans* isomerization (Figure 2.2). Exposure to light (Murphy *et al.*, 1988) thermal stress (Woolard *et al.*, 1986) and lower pH (De Ritter, 1976) are known to result in isomerization of vitamin A, the rate of which depends on the severity of the exposure. Continued exposure to light leads to further isomerization of the two predominant *cis* isomers (9-*cis* and 13-*cis*- retinyl palmitate) to give 11-*cis,* 9,11-*cis,cis,* 11,13-*cis,cis* and 9,13-*cis,cis* retinyl palmitate isomers (Murphy *et al.*, 1988). This conversion is known as photochemical isomerization, which also occurs via photosensitisers. The amount and the ratios of the *cis*-isomers vary with the means of photo-isomerisation (Fennema, 1996).

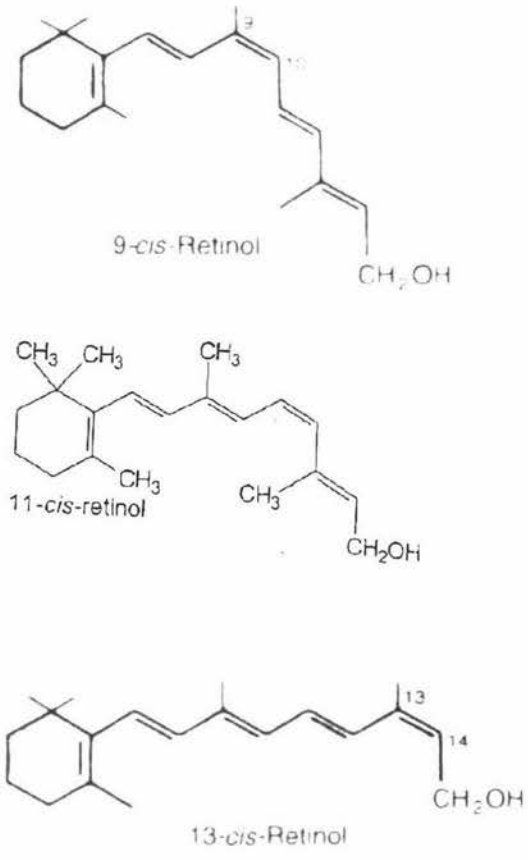

Figure 2.2 Different isomers of the vitamin A (adapted from Ball, 1998 and Bender, 2003)

Isomerization mainly affects the vitamin A activity. The predominant form all-*trans*-retinol, exhibits the greatest vitamin A activity with a biopotency of 100 IU. In contrast, the 13-*cis* isomers have ~ 75 IU of vitamin A activity.

2.3.3 De-esterification

Vitamin A degradation also occurs through the reduction of the more stable ester form to the more labile alcohol form. This process known as "de-esterification", and is primarily caused by pH values of 4.5 or below (Erdman *et al.*, 1988).

2.4 Factors affecting the stability of vitamin A

Many factors can affect the stability of vitamin A in a food system, including oxygen, light, storage and processing temperatures, pH, and the presence of other vitamins,

food components and metallic ions such as iron and copper. The most important of these factors for vitamin A stability are oxygen, light, pH and temperatures.

2.4.1 Oxygen

The combination of multiple double bonds and the polar end group make vitamin A highly susceptible to oxidation. As a result, much research has focused on the impact of the presence of oxygen on vitamin A stability (Vassila *et al.*, 2002; Lloyd *et al.*, 2004; Moyssiadi *et al.*, 2004). Oxidative degradation of vitamin A in foods can occur by direct exposure to oxygen or by the indirect effects of free radicals (section 2.2.1 and 2.2.2). Krukowsky *et al.* (1943) reported a functional relationship between rate of the influx of atmospheric oxygen and the rate of destruction of vitamin A. It appears that the oxidation produces intermediate epoxides, which consist of a ring shaped bond between 2 carbon atoms in the β-ionone ring and an oxygen molecule. Tesoriere *et al.* (1997) proposed a mechanism of retinol oxidation, which showed radical addition to the cyclohexenyl ring, resulting in the formation of 5,6-epoxide (Figure 2.3) as an intermediate oxidation product.

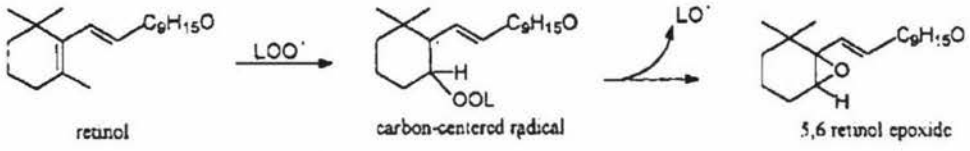

Figure 2.3 Formation of 5,6 epoxide of retinol (adapted from Tesoriere *et al.*, 1997)

Certain forms of vitamin A are more susceptible to oxidation than others, with the esterified form of vitamin A (retinol acetate and palmitate) being more resistant than the alcohol form (Ball, 1998). These retinol esters are frequently used in the vitamin A fortification of food products. The rate of oxygen-induced degradation depends on

factors such as the headspace oxygen, oxygen permeability of the packaging and the total amount of oxygen contained within the product and its environment.

2.4.1.1 Headspace Oxygen and the amount of oxygen within packaging

Reducing the amount of headspace oxygen can considerably improve the nutritional quality of milk powders through such factors as increased retention of fat-soluble vitamins (Coulter, 1947; Warmbier *et al.*, 1976; deBoer *et al.*, 1984; Lloyd *et al.*, 2004). In an early study, nitrogen flushing was used to reduce headspace oxygen levels down to 5 %. However Kacyn *et al.* (1983) reported that the presence of 5 % oxygen still resulted in vitamin A oxidation.

It has been reported that inclusion of oxygen absorbers in the packages generally lowers the headspace oxygen level to ~ 1 % (Chan, 1993). They are more successful than nitrogen flushing in reducing oxygen (Lloyd *et al.*, 2004) and thus delaying the oxidation of nutrients in whole milk powders (Chan, 1993). However, the use of an oxygen absorber can be effective only if their absorbing capacity is not wasted prior to packaging and if a sufficiently low level of headspace oxygen is maintained within the packaging (Lloyd *et al.*, 2004). However, the use of oxygen absorbers is limited to inclusion in dry food materials.

Loyd *et al.* (2004) studied 10 brands of non-fat dry milk powders obtained from retail distributors, and found that some powders had no remaining vitamin A. There was a significant variation in the oxygen content within each can, suggesting that the oxidation reactions could be responsible for the loss of vitamin A (Kacyn *et al.*, 1983). Limiting the oxygen trapped within the packaging could be useful in order to minimise vitamin A oxidation.

2.4.2 Light

Retinol and its derivatives are inactivated by UV radiation. The vitamin A degradation due to light exposure occurs either by photochemical oxidation (section 2.3.1.1) or photoisomerization (section 2.3.2). The mechanism to explain the loss of retinol due to light exposure is more complex than a first-order reaction and probably includes a combination of both isomerization and oxidation (Murphy *et al.*, 1988).

Photoisomerization reactions have been shown to compete with photo-degradation reactions, and the predominant reaction will depend on light intensity. Whited *et al.* (2002) reported a dose response of light intensity on vitamin A loss. The authors concluded the length and the intensity of the light exposure directly influence vitamin A losses.

The wavelength of light has been reported to influence the photooxidation of vitamin A (Sattar *et al.*, 1977; Fanelli *et al.*, 1985; Cladman *et al.*, 1998). Sattar *et al.*, (1977) identified certain wavelengths of vitamin A spectral absorption that result in vitamin A loss. Wavelengths < 415nm result in the destruction of vitamin A, while wavelengths between 415-455nm cause slight degradation (Sattar *et al.*, 1977). The vitamin A degradation due to light exposure was minimised when wavelengths < 455 nm were blocked, using special filters (Fanelli *et al.*, 1985). However, these experiments were only conducted on an experimental scale and may not be very practical in commercial scale applications. Fluorescent light and natural light contain wavelengths ranging between 300 - 700nm and so could induce loss of vitamin A (Whited *et al.*, 2002). This is a major concern for the food products displayed in commercial display cabinets, which are constantly subjected to fluorescent light.

2.4.3 Temperature and time

The temperature and time combination used during processing or storage can have a significant effect on retinol stability. The processing temperatures used in the food industry may be as high as 144 °C, but product is only held at this temperature for a relatively short period. Transport and storage temperatures tend to range from 4 to ~ 40 °C, depending on the product and location of storage. However, product may be held at these temperatures for extended period of time.

2.4.3.1 Processing temperature and time

Several different heat treatments are used in food processing in order to minimise the growth of microorganisms and to maximise the shelf life. These include sterilisation, pasteurisation and ultra-high temperature (UHT) treatment (Manners *et al.*, 2003). The loss of vitamin A during heat treatment appears to be primarily due to isomerization (as discussed in section 2.3.2). Panfili *et al.* (1998) confirmed the

presence of 13-*cis*-retinol isomer in heat processed milk, and reported no such isomerization occurred in raw milk that had not been subjected to thermal processing.

The effect of heat treatment on vitamin A stability depends on the severity of the heat treatment. The two most common time/temperature combinations used for pasteurisation are 62-63 °C for 30-35 minutes (low temperature, long time) and 72 °C for 15 seconds (high temperature short time (HTST)) (Manners *et al.*, 2003). It has been reported that pasteurisation does not cause significant loss of vitamin A. Panfili *et al.* (1998) reported that HTST pasteurised milk, had an average *cis:trans* isomer ratio of between 2.6 % to 6.4 %, indicating some thermal degradation of vitamin A. The *cis*: *trans* isomer ratios reported by Panfili *et al.* (1998) indicated that vitamin A was more stable during pasteurisation at 72 °C for 15 seconds compared with 72-76 °C for 15 seconds. Further, Le Maguer *et al.* (1983) used a more severe pasteurisation treatment (85 °C for approximately 16 seconds), but also concluded that vitamin A destruction was minimal in partly skimmed milk. These researchers reported 0% loss of vitamin A in fortified skim milk and 5.6 % loss in unfortified skim milk after pasteurisation.

UHT treatment results in a much higher degree of isomerization (15.7 %) (Panfeli *et al.*, 1998). The most severe heat treatment used in the study was sterilisation, which resulted in the highest *cis: trans* ratio (33.5 %). However, the high light permeability of the glass bottles used for the sterilised milk may have also contributed to this transformation, as light-induced oxidation has also been shown to convert *trans* isomers to *cis* isomers (Murphy *et al.*, 1988).

The relative soluble whey protein nitrogen (RSWPN) may also have an effect on the vitamin A loss in milk. Milk that contains higher RSWPN (15.5 %) had isomerization ratio of 2.6 % compared to the 6.4 % showed by the milk with low RSWPN (14 %). The form of vitamin A does not appear to affect the stability of the vitamin A with both natural and synthetic vitamin A showing equal vitamin A losses in pasteurised milk (Le Maguer *et al.*, 1983).

2.4.3.2 Storage temperature and time

The effect of different storage conditions on vitamin A can be difficult to interpret due to the wide variety of methods used for analysis, some of which do not consider the different isomers (Panfeli *et al.*, 1998). For example, LeMagure *et al.* (1983) used fluorometric analysis (that does not allow the identification of different retinol isomers) and reported no significant vitamin A loss in UHT milk, while Woolard *et al.* (1985), who used chromatographic methods (that allows identification of retinol isomers) reported significant losses.

Increasing the storage temperature increases the loss of vitamin A, during storage and the rate of loss appears to be independent of any prior heat treatments. LeMagure *et al.* (1983) reported that the vitamin A content of low fat UHT milk was relatively stable during storage at 4 °C and 20 °C for up to 3 months. However, increasing the storage temperature to 35 °C resulted in 25-40 % increase in vitamin A degradation. Vidal-Valverde *et al.* (1992) reported rapid decrease in retinol content of UHT milk stored at temperatures of 30 °C compared to 40 °C.

The rate of vitamin A degradation is higher during the first few weeks of storage and seems to diminish over time. The association between the storage time and vitamin A may be summarised as " longer the storage time, greater the vitamin A degradation" (Lau *et al.*, 1986). Woolard *et al.* (1983) and DeBore *et al.* (1984) investigated vitamin A stability in dry fortified whole milk and non-fat milk powders (stored at ambient temperature for 16 weeks), respectively. DeBore *et al.* (1984) found rapid loss of vitamin A during the first 10 weeks of storage. This loss continued at a reduced rate as time progressed. Woolard *et al.* (1983) monitored the vitamin A concentration in milk powder stored at ambient temperatures for 24 months and reported similar observations. Vitamin A degraded faster over the first 10 months but remained at a reduced rate within the next 14 months of the storage in whole milk whereas the vitamin A was completely degraded after 6 to 12 months in skim milk powder. The researchers suggested a possible protective effect of naturally available fat in whole milk on vitamin A, while Woolard *et al.* (1985) hypothesized that retinyl esters continue to oxidise until oxygen is depleted. The protective effect of fat on vitamin A will be discussed in detail in section 2.4.5.

Storage at lower temperatures also showed a similar degradation trend. The rate of vitamin A degradation at refrigeration temperatures appeared to be faster at the beginning of storage, continuing at a reduced rate, after the initial losses (Vidal-valverde *et al.*, 1992). In contrast, Le Magure *et al* (1983) found that there was minimal loss of vitamin A in UHT milk for the first 12-16 weeks of storage, but the vitamin degraded rapidly after this period. This initial lag phase was attributed to the effective scavenging of residual oxygen initiated by the oxidation of sulphydryl groups, which were released by denatured α-lactalbumin and β-lactoglobulin during processing. Many of the results reported for pasteurised milk appear to be based on measuring the vitamin A content of opened packages, therefore oxygen content may have been a confounding factor. Le-Maguer *et al.* (1983) studied the effect of storage temperature and time on pasteurised low fat milk in opened paper cartons. It was found that storage at 4 °C for 12 days resulted in the degradation of between 0.6 to 7.2 % of the vitamin A. Similarly, Nakai *et al.* (1983) focused on fortified pasteurised 2 % low fat and skim milk obtained from a retail market, which had been stored there for 4 months. The opened milk packages that were stored at 4 °C showed significant changes of vitamin A during the first 4 days, but no significant losses between 4-12 days after opening.

The effect of frozen storage on vitamin A losses has also been investigated. Vidal-valverde *et al.* (1992) found no loss of vitamin A in UHT whole milk during frozen storage (-20 °C) for up to 2 months, but retinol loss occurred between 17-18 % over 4-8 months. This indicates vitamin A degradation may still occur even at very low temperatures during lengthy storage periods. These researchers suggested certain enzymes (lipases and oxidases) that survive through the UHT process might be responsible for the vitamin A losses. However, no antioxidants were incorporated into these samples, so vitamin A losses may possibly be attributed to oxidation.

The form of vitamin A (natural or synthetic) is also associated with the stability of the vitamin A in a food system. For example, in milk the synthetic form of vitamin A is associated with the serum phase, which enhances the contact with oxygen, and

reduces the stability of the vitamin. Synthetic vitamin A loss was significant with increased temperature and time particularly in low-fat milk (Lau *et al.*, 1986).

2.4.5 pH

Retinol is stable in alkali conditions but sensitive to acidity. Lower pH values have been shown to convert the predominant all-*trans*-retinol isomers to *cis*-isomers with partial isomerization of vitamin A occurring at pH 4.5 or lower (De Ritter, 1976). Low pH values may also result in de-esterification of vitamin A esters to produce the more labile free retinol (Erdman *et al.*, 1988). This may create problems for the stability of retinol in dairy-based products, such as fruit juices (fortified with whey-proteins) which usually have relatively low pH (3.5-4).

Carbon dioxide (CO_2) based acidification (achieved by bubbling CO_2 through the product) reduces the pH of milk and has been shown to be an effective inhibitor of microorganisms. This method also appears to be relatively effective at preventing retinol losses in milk reasonably well despite the pH changes. Sierra *et al.* (1996) studied vitamin A stability in CO_2-treated and untreated raw milk stored at 7 °C for 7 days. No significant differences in all-*trans*-retinol content were observed between the untreated milk (pH 6.83) and CO_2 treated milk (pH 6.0 and 6.4). It was concluded that the CO_2 acidification did not affect the vitamin A retention during cold storage. However, there was a significant increase (200 %) in 13-*cis*-retinol (which is less biopotent than the added all-*trans*-retinol) during the period of storage indicating the possible isomerization of all-*trans*-retinol. Ruas-Madiedo *et al.* (1988) reported CO_2 addition inhibited microbial growth without affecting the stability of vitamin A in pasteurised whole milk, stored at 4 °C for 4 days. The pH of milk was reduced from 6.72 to 6.24 through acidification. The CO_2-treated milk showed slightly higher retinol levels than the controls, suggesting a possible mild protective effect of the CO_2.

2.4.5 Effect of fat content

A protective effect of the presence of fat on vitamin A stability in foods has been reported in the literature. The photooxidative stability of vitamin A was higher in milk containing 2 % fat than skim milk (Fellman *et al.*, 1991; Whited *et al.*, 2002). Sattar *et al.* (1977) studied the effect of fat content on light sensitivity of vitamin A and reported that the loss of vitamin A is inversely proportional to the thickness of the fat layer that the vitamin is associated with. Increased fat content in milk reduces the light penetration through the milk and hence reduces the loss of vitamin A (Fellman *et al.*, 1991).

The presence of fat also appears to protect vitamin A with regards to temperature. Lau *et al.*, (1986) reported a protective effect of fat content on vitamin A with regard to time and temperature in UHT milk. The researchers compared the vitamin A degradation of UHT milk with fat contents of 0.15, 2.92, 6.16 and 9.70 % and found that the final vitamin A concentration at the end of 3 weeks of storage was higher in milk with high fat content. It is known that the vitamin A naturally present in milk is associated with the fat globules, meaning that an increase in milk fat will also increase the vitamin A content of milk sample.

2.5 Methods for stabilising vitamin A

The technologies used to stabilise vitamin A in food systems vary from external physical barriers to technologies that aim to interfere with intrinsic vitamin A degradation modes. The external factors used to stabilise vitamin A in food systems are mainly different packaging materials in conjunction with the use of colour pigments, UV absorbers and labels. The internal protectors include antioxidants, emulsions, encapsulation and protein binding.

2.5.1 Packaging materials

Different packaging materials that have been tested for the light stability of vitamin A include polyethylene terepthalate (PET) bottles (clear and containing colour pigments), low-density polyethylene (LDPE) pouches and high-density polyethylene (HDPE) bottles and jugs (clear and containing colour pigments). These studies have

investigated the effect of colour pigmented packaging material on the stability of the vitamin A in whole milk, low fat milk, and skim milk. Cladman *et al.* (1998) reported that the use of green-coloured PET bottles resulted in lower vitamin A losses in whole milk than clear PET bottles. The vitamin A content of low fat milk in green PET bottles showed less degradation than in clear PET bottles, HDPE jugs or LDPE pouches (Cladman *et al.*, 1998). In addition, PET with titanium dioxide (TiO_2) effectively prevented vitamin A against light degradation (Moyssiadi *et al.*, 2004).

Fanelli *et al.* (1985) tested the effectiveness of the yellow, red and blue pigments that are being compounded into the polyethylene resin that is used to produce HDPE bottles. The researchers found that the 5-oxo-1-(parasulfophenyl)-4-(parasulphophynilalso) 2-pyrazoline-3-carboxylic acid on Al_2O_3, which was identified as the yellow pigment, protected the vitamin A in whole milk. The researches superimposed the absorption spectra of the vitamin A and the yellow pigment, demonstrating the ability of the yellow pigment to absorb the critical 300-400 nm wavelength regions. However, the researchers also speculated that vitamin A protection might have been affected by non-specific background absorption. Moyssiadi *et al.* (2004) evaluated vitamin A stability in low fat milk stored in pigmented monolayer and multilayer HDPE bottles and reported vitamin losses of 11 % and 16 % respectively. This implies that the multilayer HDPE may be more effective in retaining the vitamin A in low fat systems. Vassila *et al.* (2002) studied the vitamin A retention in whole milk stored in 3 different LDPE pouches (clear, pigmented with either TiO_2 (white) or Carbon (black) and pigmented with both TiO2 and Carbon). The researchers found a higher degree of vitamin A loss (50.9 % - 73.6 %) in clear LDPE pouches and the pouches pigmented with only a single colour pigment, whereas relatively low vitamin A losses were shown (15.1 %-18.9 %) in LDPE pouches containing both TiO_2 and Carbon. The thickness of these pouches also may have contributed to these low losses of vitamin A, as the pouches with both pigments were thicker (110μm), when compared to the clear and single pigmented (60μm) pouches.

UV absorbers may absorb light below 400nm and thus protect vitamin A from degradation. Cyasorb531 (2-OH-4-n-octoxybenzophenone) and Tinuvin 326 (2-(3`-t-butyl-3`-OH-5`-methylphynel)-5-chlorobenzotriazole) are approved for contact with

food and have demonstrated a stabilising effect for vitamin A in milk (Fanelli *et al.*, 1985).

It is possible to combine UV blockers and pigments in order to further reduce vitamin A degradation. Cladman *et al.* (1998) reported significantly lower vitamin A losses in UV-absorbing PET bottles. However, the researchers did not monitor or maintain consistent oxygen permeability of the packaging material, therefore oxygen may have affected the final vitamin A level in the samples due to possible oxidation.

The potential use of a label to shield the milk in bottles from light has been suggested (Hoskin, 1988; Cladman *et al.*, 1998). Hoskin *et al.* (1988) observed that shielding large portion of the sides of a milk bottle is more effective than shielding from the top (Figure 2.4) and recommended that the shielding material be printed in a dark colour to minimise light transmission. Cladman *et al.*, (1998) studied the effect of a label, which covered 55 % of the surface of the bottle and reduced visible light transmission by 75 %. The researchers reported a 20-30% reduction in vitamin A losses in labelled bottles compared to non-labelled bottles.

Packaging used for UHT treated food products is also effective in improving vitamin A stability. UHT packaging is sterile and impermeable to light and oxygen, although there is minimal amount of oxygen trapped in the airspace during closure (Woolard *et al.*, 1985).

The oxygen permeability of the packaging materials can have a considerable affect on the vitamin A stability (Vassila *et al.*, 2002). Significantly lower oxygen transmission rates for the low-density polyethylene (LDPE)/polyamide (PA)/LDPE sandwiched packaging may mean this packaging material may offer benefits in vitamin A protection through its reduced oxygen permeability and reduce oxidative damage to vitamin A.

Figure 2.4 Shielding larger portion of the side of the bottle is more effective than shielding from the top (Hoskin *et al.*, 1988).

2.5.2 Antioxidants

Antioxidants protect vitamin A by intercepting oxygen molecules, before they react with the vitamin. The antioxidant is oxidised in the process, thus the antioxidant capacity of the system will be depleted over time until the vitamin becomes vulnerable to oxygen. The limited use of oxygen absorbers mentioned in the section 1.3.1.1 can be overcome by using food grade antioxidants such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) that have been shown to protect vitamin A degradation in solid and liquid food products. BHT has been often used as an antioxidant in studies of vitamin A and milk proteins (Manderson *et al.*, 1998; Considine *et al.*, 2005). The addition of 0.05 % of BHT or BHA improved the vitamin A retention in semi-solid corn oil mixture for up to one year (Halbaut *et al.*, 1997). However, contradictory findings have also been reported. For example antioxidants such as α -tocopherol have been reported to be ineffective at stabilising vitamin A in milk (Berge *et al.*, 1987).

2.5.3 Encapsulation

The stability of vitamin A can be improved by encapsulation, which incorporates retinoids into carrier systems or particles. Such carrier systems have shown the ability to protect retinol against oxidation, particularly in dairy products. Halbout *et al.* (1997) reported oxidative degradation is reduced when semi-solid excipient mixtures with corn oil were used to encapsulate vitamin A palmitate. The researchers also found that vitamin A loss was greater in a semi-solid mixture containing 45 % corn oil (A) than a mixture containing 25 % corn oil (B). These results were attributed to the corn oil being very sensitive to oxidative degradation. However, in the presence of antioxidants BHT and BHA (0.05 %), corn oil base A retained 68 % vitamin A compared to 76.6 % of corn oil base B. It is important to note that using corn oil for encapsulation will increase the fat content of the product, which may reduce consumer acceptance.

Beaulieu *et al.* (2002) studied the physicochemical characteristics of whey protein beads formed by emulsification followed by Ca^{2+} induced cold gelation of pre-denatured whey protein. These beads had an excellent capacity to encapsulate vitamin A at 20 % (w/w) $CaCl_2$ and it was hypothesized that whey protein beads would have the potential to protect fat-soluble molecules such as retinol from oxidation.

2.5.4 Emulsions

Emulsions provide an option for fortifying aqueous-based food products with vitamin A carried in lipid droplets. Zahar *et al.* (1986) investigated the effect of type and amount of retinyl palmitate carriers such as butter, coconut, and corn or peanut oils. The carrier oils were dispersed in a fine emulsion by homogenisation. Butter and coconut oil demonstrated better vitamin A retention over 96 hours than corn or peanut oil. Corn and peanut oils contain higher levels of unsaturated oils that may undergo autooxidation (Nawar, 1996), leading to the degradation of the entrapped retinyl palmitate. However, consumer acceptance of fat in food products is generally higher for unsaturated than saturated fats.

It is also possible that the β–carotene that is naturally present in butter oil could have reduced vitamin A degradation, as the synergetic action of carotenoids and retinol in stabilising vitamin A in fortified milk has been reported (Zahar *et al.*, 1992). The protective effect of β-carotene is attributed to its ability to act as a quencher, neutralising sensitisers in the oxidation pathways (Min *et al.*, 2002).

2.5.5 Protein binding

It is known that retinol can bind to a variety of proteins. Retinol transportation within the human body is facilitated by a protein called retinol-binding protein (RBP) (Bender, 2003). Retinol is associated with lipoproteins in food matrixes (Ball, 1988), and binds to a number of whey proteins such as β-Lg (Kontopidis *et al.*, 2002) and BSA (Futterman *et al.*, 1972). Binding to a protein restricts the mobility of retinol and thus may prevent the molecule engaging in reactions (such as isomerisation), which would result in its degradation. This reduction in mobility results in an increase in fluorescence of retinol molecule (Futtreman *et al.*, 1972). Fugate *et al.* (1980) studied the spectroscopic characteristics of the retinol-β-Lg complex, and reported that the binding site enforces rigidity on the retinol molecule, especially the conformation of the β-ionone ring relative to the polyene chain. β-Lg has also been shown to be a carrier of lipophilic nutrients, in particularly fat-soluble vitamins (Swaisgood *et al.*, 1998).

BSA has also been shown to inhibit light-induced degradation (Futterman *et al.*, 1972). These interactions between the milk proteins and vitamin A raise the possibility of using these proteins as carrier molecules for hydrophobic vitamins. The nutritional significance and solubility of whey proteins makes them an excellent candidate for food fortification purposes as they will add protein and not fat to the food product.

2.6 Whey Protein Isolates (WPI)

WPI is essentially manufactured by concentrating and drying whey (Walsh, 2002). The process often involves ion exchange adsorption followed by concentration and spray drying. WPI production by ion exchange adsorption is based on the isoelectric

point (pH 4.6) of the whey. At pH values lower than 4.6, whey proteins have a net positive charge and thus behave as cations, allowing them to be adsorbed on cation exchangers (Mulvihill *et al.*, 1992). At pH values above the isoelectric point, whey proteins exhibit a net negative charge and behave as anions, thus can be adsorbed by anion exchangers. These two major ion exchange fractionation processes have been used for commercial manufacture of WPI. The adsorption processes recover ~ 85 % of the protein, which is further purified by ultrafiltration and is then spray dried. These processes allow the separation of lactose and minerals from the whey fraction of milk.

WPI usually contains >90 % protein (Walsh, 2002), with β-Lg, α-L, BSA, and immunoglobulins being the main constituents. The β-Lg and α-La have the most significant functional properties of the WPI, especially with regard to their emulsifying, foaming, hydration and gelation properties, while some whey proteins have the ability to bind to retinol.

2.6.1 β-Lactoglobulin

The amino acid sequence of the β-Lg primary polypeptide chain was first published in 1979 (Braunitzer *et al.*, 1979). It is a globular protein that consists of 162 amino acids and has a molecular weight of 18 kDa. The two disulphide bonds occur at the cysteine residues 106 and 109 while a free cysteine residue at 121 occurs in the native protein, which becomes active in the unfolded protein structure that results during denaturation (Zhang *et al.*, 2002). The secondary structure of the β-lg consists of 10-15 % α-helixes, 51 % β-sheets and 47 % of unordered structures (Fox, 2003). Crystallographic, NMR and X-ray studies have shown that β-Lg contains 9 strands of β-sheets identified as A to H (Sawyer, 2003). Eight anti-parallel β-sheets form a calyx, which is flanked on one side by an α-helix, forming a hydrophobic pocket inside (Dufour *et al.*, 1991). Crystal structures (Figure 2.5) have shown that disulphide bridges link residue 66 to 160 and 106 to 109 (Qin *et al.*, 1998). The structure varies from monomer to dimer to octamer, depending on the pH and temperature, e.g it is a monomer below pH 3.5 and above pH 7.5, while it appears as a dimer within pH 5.5 - 7.5 (Sawyer, 2003). β-Lg displays an octomeric structure between the pH values of 3.5 to 5.5, while above pH 8.6 β-Lg undergoes

polymerisation (Kontopidis *et al.*, 2002). However, under physiological conditions, the structure also depends on other factors, such as ionic strength and protein concentration (Zhang *et al.*, 2002).

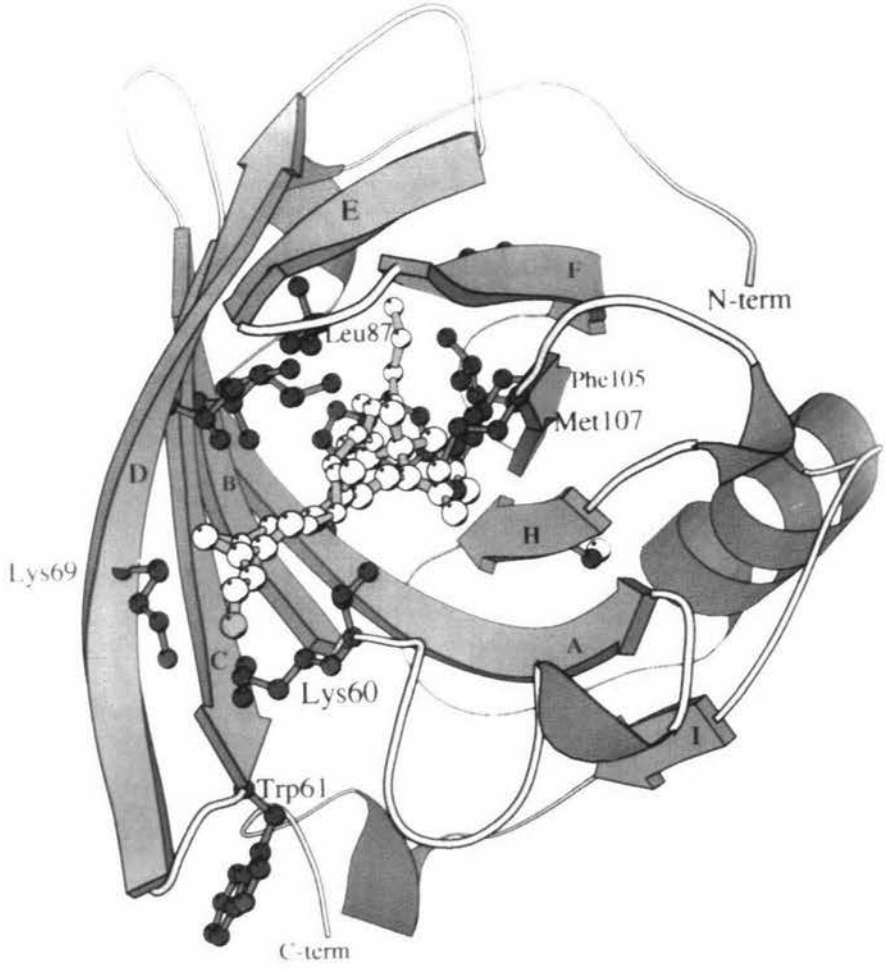

Figure 2.5 The ligand binding sites of β-Lactoglobulin. Lys 6o and Lys 69 mark the opening of the central calyx where polar head groups can interact with. Retinol binds at Phe 105 and Met 107 must move to accommodate retinol (Adapted from Fox, 2003).

2.6.1.1 Interactions of β-Lg with retinol

In vitro studies have shown that small hydrophobic molecules, including retinol, can bind to β-Lg. The ability of β-Lg to bind retinol and form water-soluble complexes was first reported by Futterman *et al.* (1972). Fugate *et al.* (1980) studied the β-Lg-

retinol complex using fluorescence spectroscopy, and found the binding site is located in the hydrophobic region of the molecule. The exact retinol-binding site has been the subject of debate for the past decade. β-Lg has two hydrophobic pockets that have the potential to bind hydrophobic molecules (Wang *et al.*, 1999). Monaco *et al.* (1987) reported that hydrophobic pocket is in between the α-helix and the surface of the β-barrel, while Papiz *et al.* (1986) reported it was in the calyx formed by the anti-parallel β-sheets. The latter was shown by X-ray crystallography and was supported by Narayan *et al.* (1997), Wang *et al.* (1997), Qin *et al.* (1998), Wu *et al.* (1999). Cho *et al.*, (1994) showed that retinol binds to the interior cavity using site-directed mutagenesis. The results of Monaco *et al.* (1987) are likely to reflect a possible second hydrophobic binding site (Wang *et al.*, 1999) and prior occupancy of the preferred binding site inside the centre hydrophobic cavity by another molecule (Dufour *et al.*, 1990; Cho *et al.*, 1994). Recently, crystallographic evidence has confirmed that the binding site at pH 5 is located in the central hydrophobic cavity (Kontopidis *et al.*, 2002).

It has been speculated that Lys60 and Lys69 (which are at the opening of the ligand-binding cavity) may play a significant role in the ligand affinity. Song *et al.* (2004) demonstrated that the strand D of β-Lg (residue 66 to 76) participates in the retinol binding with the use of epitope mapping of a monoclonal antibody specific to bovine β-Lg. This supports the potential role of Lys60 and Lys69 residue in binding to retinol. Kontopidis *et al.*, (2002) produced crystallographic evidence of the hydroxyl group of retinol being closely located close to the amino group of the Lys69 residue. The failure of pig β-Lg (where the Lys69 is substituted with Glu) to bind to retinol supports the role of Lys69 in the binding (Qin *et al.*, 1998); however, the lack of binding may be due to more complex reasons than just the substitution of the amino group(Considine *et al.*, 2005). In contrast, Papiz *et al.* (1986) and Cho *et al.* (1994) reported the possible role of the Lys70 residue in the binding of retinol to β-Lg, while Fugate *et al.* (1980) suggested that binding site is likely to include the Trp19 residue. Papiz *et al.* (1986) also indicated that the Trp19 of β-Lg is essential for retinol binding. However, Katakura *et al.* (1994) concluded that the Trp19 is not essential for binding but is relevant to stabilising the bound retinol.

Extensive research has been carried out looking at the affinity of retinol towards the binding site. Fugate *et al.* (1980) reported that retinol binds to β-Lg with an affinity similar to the retinol binding protein (which is homologous to β-Lg). It is important to note that different methodologies have been used for the affinity studies, including solubility, affinity chromatography, equilibrium dialysis and fluorescence. The fluorescent titration method is based on the fluorescence emission of the Trp19 (located at the bottom of the hydrophobic cavity (Papiz *et al.*, 1986) upon binding to retinol (Cho *et al.*, 1994). Fugate *et al.* (1980), used the fluorescent titration method to show an association constant ($K`_a$) of $2 \times 10^{8} M^{-1}$ at saturation, which is consistent with the results of affinity chromatography studies by Jang *et al.* (1990). In contrast, another study (Puyol *et al.*, 1991) using gel filtration and equilibrium dialysis reported the $K`_a$ of retinol-β-Lg as $1.5 \times 10^{4} M^{-1}$, which is about four orders of magnitude less (Perez *et al.*, 1995). These discrepancies in the results may have resulted from the different techniques as well as the different genetic variants of β-Lg used in the study (Considine *et al.*, 2005). Dufour *et al.* (1991) interpreted the association of retinol to β-Lg in terms of dissociation constant ($K`_d$) due to the possible existence of unbound ligand in the equilibrium. The findings suggested that the binding constant was influenced by the pH. The reported retinol-βLg $K`_d$ was 4.4×10^{-8} M at pH 7 which was in good agreement with Wang *et al.* (1997). Dufour *et al.* (1994) used fluorescence spectroscopy and found a $K`_d$ of 8.3×10^{-8} at pH 3 and 2.8×10^{-8} at pH 8, which indicated weaker association of retinol with β-Lg at the higher pH. Another study which focused on the Trp19 fluorescence quenching of β-Lg isolated by bio-selective adsorption, and showed that affinity decreased about four fold as the pH was lowered towards 5.5 (Wang *et al.*, 1997). However, Fugate *et al.* (1980) used the fluorescence titration method and reported that the $K`_d$ value was 2×10^{-8} M and was pH independent in the range of pH 2 to 7.5.

Chemical modifications of β-Lg, such as methylation, alkylation (Dufour *et al.*, 1990) and esterification (Dufour *et al.*, 1993; Dufour *et al.*, 1994), enhance its binding affinity for retinol. Dufour *et al.* (1990) suggested that methylation, which does not dis-assemble the ligand-binding site, might possibly open up a second binding site for retinol. The researchers also suggested that the increased hydrophobicity of the surfaces of the methylated and ethylated β-Lg might also explain the increased

affinity. On the other hand, esterification of β-Lg carboxyl groups strengthens the β-Lg interaction with retinol and accelerates the beta-strand to alpha-helix transition.

In vitro studies have shown that one retinol monomer binds tightly to one β-Lg molecule (Futterman *et al.*, 1972; Dufour *et al.*, 1994). Fugate *et al.* (1980) reported that retinol binds to both monomeric and dimeric β-Lg with similar binding constants. However, the stoichiometry of β-Lg and retinol binding appears to depend on the pH value of the solution. Wang *et al.* (1997) reported this stoichiometry as 1.0 mol mol^{-1}, however, this changed and fluctuated between 0.65 and 0.82 $mol.mol^{-1}$, in low pH systems, indicating possible protein denaturation or binding sites being already occupied with lipids. As pH decreased the affinity of retinol to the β-Lg also decreased, but there was no change in the stoichiometry of binding. However, changes of pH in the presence of alcohol did affect the stoichiometry with Dufour *et al.* (1990) reporting that retinol-β-Lg stoichiometry doubles when β-Lg is in 20% ethanol at pH 6.

2.6.1.2 Effects of retinol on the stability of β-Lactoglobulin

Bovine β-Lg can be denatured by several factors including heat, organic compounds, metal ions and pressure. Denaturation of β-Lg is triggered by the initial dissociation of dimer to monomer, which leads to conformational changes in the polypeptide chain and subsequent aggregation (Sawyer, 2003).

Reddy *et al.* (1988) reported that heating at 50, 60, and 70 °C for up to 15 minutes did not affect the β-Lg conformation, while measurable changes were observed at 80-90 °C. Higher temperatures caused disulfide bond interchanges followed by partial unfolding of β-Lg and subsequent exposure of thiol group, Cys 121 (Manderson *et al.*, 1998). Dimer dissociation occurs between 30 °C-55 °C (Sawyer, 2003) while destruction of the α-helix (which covers the Cys 121) occurs between 60 °C-70 °C (Qi *et al.*, 1997). Considine *et al.* (2005) used circular dichroism (CD), fluorescence and gel electrophoresis to study the effect of retinol binding on the heat stability of β-Lg (40-93 °C for 12 minutes). As the temperature increased, considerable amounts of non-native small oligomers were observed in the retinol-bound β-Lg, while changes

in CD patterns indicated a protective effect of retinol on β-Lg denaturation at temperatures close to 70 °C. It is important to note that the heat-induced denaturation of β-Lg also depends on the pH and protein concentration (Sawyer, 2003). The effect of retinol binding on β-Lg stability with regards to the protein concentration has not been studied.

Organic compounds including urea, guanidinium hydrocloride (GuHCl) and alcohol cause the denaturation of β-Lg. Hattori *et al.* (1993) reported a complete loss of retinol binding activity of β-Lg in the presence of 6M GuHCl. However, the denaturation appeared to be reversible with the use of exhaustive dialysis to remove the denaturant. Renatured β-Lg regained the binding activity of retinol as strongly as the native β-Lg (Hattori *et al.*, 1994). Similarly, concentrations between 4-5 M of urea cause β-Lg denaturation, and the kinetics of this reaction have been shown to be pH dependent (Sawyer, 2003). Binding of palmitate to β-Lg has been proven to stabilise the β-Lg against urea denaturation (Creamer, 1995), although the effect of retinol (itself) binding on urea denaturation has not been studied,. Interestingly, ethanol also unfolds the β-Lg but the reaction is not completely reversible (Sawyer, 2003). Retinol binding has no effect on folding of β-Lg induced by alcohol (Dufour *et al.*, 1993).

β-Lg has shown to increase the gastrointestinal retinol uptake. Said *et al.* (1989) showed increased retinol uptake in the rat intestine when retinol was bound to β-Lg, while Puyol *et al.* (1993) studied the effect of retinol binding on trypsin digestion and found that retinol has no impact on the β-Lg proteolytic resistance. This supports the proposed role of β-Lg as a retinol carrier. However, β-Lg denatures in alkali conditions and little work has been done on the effect of increasing pH (Sawyer, 2003). No studies on the effect of retinol on β-Lg stability under alkali conditions have been reported.

2.6.2 α-Lactalbumin

α-Lactalbumin (α-La) is a relatively small molecule with an approximate molecular weight of 14 K Da. The isoelectric point of α-La is pH 4.8 (Fox, 2003), which

indicates that it is slightly acidic. Bovine α-La contains 123 amino acids (Brew, 2003). The three-dimensional structure of the α-La has been produced by X-ray crystallography by Acharya *et al.* (1991): it exists as a compact globular structure that is also known as a prolate elliposoid (Brew, 2003), which consists of an α-lobe formed by segregated α-helices and a β-lobe formed by β-sheets as well as unordered structures. The disulphide bond between the Cys73 of the β-lobe and Cys90 of the α-lobe is one of the 4 intra-molecular disulphide bonds in α-La. Two of the remaining disulphide bonds (those linking Cys6 to Cys120 and Cys28 to Cys111) are in the α-lobe, while the β-lobe contains the remaining disulphide bond between Cys60 and Cys77.

The functional value of α-La is very important as it is the regulatory protein of the lactose synthase enzyme that catalyses and regulates lactose synthesis. This is clearly reflected by the lactose concentration of milk, which is directly related to the α-La concentration (Brew, 2003). α-La also promotes the binding of glucose to galactosyltransferase, which catalyses the final step in lactose biosynthesis (Kronman, 1981).

α-La can be denatured by several factors including pH, heat and organic compounds (i.e. urea). Under acidic conditions, α-La undergoes a trans-conformation to a non-native state known as the molten globe state (Brew, 2003). This molecular state still retain the globular shape of the molecule but is attached to 270 water molecules and is subsequently swollen (Permyakov *et al.*, 2000).

2.6.2.1 Interactions between retinol and α-La

Using gel filtration, Puyol *et al.* (1991) found that α-La binds retinol *in vitro*, and that the percentage of retinol bound to α-La (36%) was much higher than that bound to β-Lg. Hirai *et al.* (1992) suggested that small lipophilic compounds, which bind to α-La may be protected or stabilised. However, α-La is less abundant in the whey fraction than β-Lg, and is unlikely to be responsible for binding as much of a ligand as

the β-Lg in a whey protein solution despite the higher binding capacity of the α-La molecules.

2.6.3 Bovine Serum Albumin (BSA)

BSA has a molecular weight of 66 KDa (Fox, 2003). Hirayama *et al.* (1990) reported the correct amino acid sequence, which contains 583 amino acids, although previously the amino acid sequence was thought to contain 582 amino acids (Farrell *et al.*, 2004). It contains the highest number of cysteine residue (35 per molecule) among all the whey proteins (Walsh, 2002). These cysteines are linked by 17 disulphides, which create two loops (Hsia *et al.*, 1984). These two loops account for two out of the three domains of the BSA molecule, while a shorter chain accounts for the third domain. The N-terminal and C-terminal are located in different domains.

2.6.3.1 Interactions between retinol and BSA

Futterman *et al.* (1972) reported that BSA forms strongly fluorescent water-soluble complexes with retinol with a molar ratio of ~ 1. They also observed that the fluorescent yield of retinol complexed with BSA was about 4 times larger than the retinol alone in petroleum ether. Puyol *et al.* (1991) used gel filtration to study the interactions between whey protein and retinol, and also observed the ability of BSA to bind to retinol. However, the researchers concluded that retinol transportation was not a major functional property of BSA, although these results indicate that this could be a possible physiological role of BSA. BSA is immunologically and physiologically identical to the human blood serum albumin, and recently Fortuna *et al.* (2003) reported that circulating retinol is associated with both the human retinol-binding protein (RBP) and BSA. However, no *in vitro* studies have yet shown evidence to support this association.

2.6.3.2 Factors affecting ligand binding and stability of BSA

Ligand binding to BSA is affected by extrinsic factors, such as pH, and intrinsic factors, such as ionic charge and polarity. The results of Spector *et al.* (1969) indicated that the maximal binding of palmitate to BSA required the presence of positively charged protein sites. However, Anderson *et al.* (1971) suggested that

some polar lysine or arginine residues, which are situated at the clefts between the globular parts of the BSA, may create a favourable environment for ligand binding. Binding properties of BSA appear to be affected by the pH of a solution due to the possible structural alterations of the protein. Binding of free fatty acids was reduced when BSA was acetylated at pH 5 or treated with formaldehyde at pH 11, and binding was almost abolished when BSA was acetylated at pH 8 (Spector *et al.*, 1969).

Binding of ligands appears to stabilise BSA as they mask the hydrophobic sites of the BSA. Anderson *et al.* (1971), who studied the kinetics of the reactions between trinitribenzenesulfonic acid (TBNZ) and BSA, suggested that the binding of free fatty acid anions at the clefts could diminish the freedom of movement of the subunits of the protein versus each other and therefore the long chain fatty acids could stabilise BSA against heat denaturation as well as against proteolytic digestion.

2.7.4 Immunoglobulins

Immunoglobulins (Ig) are antibodies synthesised in response to stimulation by antigens foreign to the animals. There are five classes of immunoglobulins present in bovine serum and are known as IgA, IgI, IgD, IgM and IgG. All these have a similar basic structure, which is composed of four polypeptide chains covalently linked by disulphide bridges with a molecular weight of approximately 160,000 Da (Larson, 1992). These exist as polymers or monomers, while IgG the predominant class of Ig, always exist in monomeric form. IgG comprise ~ 80% of the total Ig and has two identical light chains and heavy chains, which differ in amino acids but have homologous sequence (Larson, 1992). Immunoglobulins are among the most heat sensitive whey proteins and can exert antimicrobial action.

It appears that there could be link between the amount of Ig secreted and the vitamin A deficiency in a milk-producing animal. Nonnecke *et al.*, (1993) reported retinoids modulate IgM secretion by cultures of bovine mononuclear leukocytes. However, specific interaction between the Ig and retinol is yet to be elucidated.

2.7.5 Other whey proteins

Lactoferrin, transferrin, β_2-microglobulin, proteose-peptone fractions and a group of acid glycoproteins are also present in the bovine whey. Lactoferrin and transferrin appear to be large single chain polypeptides and the reported molecular weights vary from 75,000 to 77,000 Da for the transferrin and from 77,000 to 93,000 Da for the lactoferrin (Walstra, 1984). These proteins are known as iron-binding proteins and thus biological function of these proteins is the transportation of iron.

The molecular weight of β_2-microglobulin is known to be 11,800 Da and it consists of a single polypeptide chain of about 100 amino acids. Its amino acid sequence indicates homology with the constant regions of Ig (Walstra, 1984) and the function of this protein is yet to be elucidated (Larson, 1992).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Whey protein isolate (WPI 895) was obtained from Fonterra Co-operative Ltd, Palmerston North. WPI solutions ranging from 5-10% (w/v) were prepared in phosphate buffer containing 26 mM Na_2HPO_4 and 68 mM NaCl. Approximately 0.02% (w/v) of sodium azide was added to prevent microbial growth. All-*trans*-retinol (R-7632) and retinol acetate (R-4632) were sourced from Sigma Chemical Co. (St. Louise, MO). All reagents used for High Performance Liquid Chromatography (HPLC) analysis were HPLC grade, and reagents for sample preparation were analytical grade. MilliQ water (MilliQ system, milliporeCorp., Bedford, MA) was used for all experiments. All experimental procedures were carried out in the absence of light or with minimum light exposure ($\leq$ 13 lux (lx)). To negate the effect of light, all glassware containing samples/ controls or standards (Table 3.1) were wrapped in aluminium foil at all times.

Table 3.1- Contents of the standards, controls and samples

Name	Contents
Standard	Ethanol, Retinol
Control-I	Buffer, Ethanol, Retinol
Sample	Buffer, Ethanol, Retinol, WPI,
Control-II	Buffer, WPI

3.1.1 Retinol standards

In order to pre-test the separation capacity of the HPLC, 10 mg of all-*trans*-retinol and an equimolar amount of BHT were measured in the dark and made up to 10 ml with ethanol to obtain 1 mg/ml retinol stock solution. This solution was diluted with ethanol to prepare standards ranging from 10 to 500 µg/ml. Standards were filtered through 0.45 µ Millipore® filters sourced from Sartorius- viva science (AG-30625,

Hannover, Germany) and analysed by the HPLC as described in section 3.3. The resulting chromatograms showed flat-topped peaks for retinol concentrations greater than 300 μg/ml indicating the absorbance of such concentrations was too high for the detection method used and thus retinol concentrations ranging from 10 to 300 μg/ml were used for all experiments.

3.1.2 Preparation of controls and samples

Stock solution of retinol was prepared by measuring 75 mg of all-*trans* retinol and an equimolar amount of BHT in the dark and making to 15 ml with ethanol to obtain a final concentration of 5 mg/ml. The stock solution was diluted with phosphate buffer to prepare control-I (Table 3.2). Samples (Table 3.3) were prepared with 1% WPI and various retinol concentrations (10-300 μg/ml). The pH values of the samples were checked to ensure sufficient buffering capacity. In order to study any possible interference from WPI, the control-II was prepared with 1% of WPI in buffer and no retinol. The standards were prepared with retinol in ethanol (Table 3.4). The samples, standards and controls were stored at 5 °C in the dark until solvent extraction for analysis.

Table 3.2 The composition of control-I: (retinol in aqueous buffer), with a total volume of 50 ml.

Retinol* (ml)	Absolute ethanol (ml)	Phosphate buffer (ml)	Retinol concentration in the sample (μg/ml)
0.1	5.9	44	10
0.5	5.5	44	50
1	5	44	100
2	4	44	200
3	3	44	300

* Retinol stock solution 5mg/ml

Table 3.3 The composition of the samples containing 1% WPI with a total volume of 50 ml

5% WPI (ml)	Retinol* (ml)	Absolute ethanol (ml)	Phosphate buffer (ml)	Retinol concentration in the sample (μg/ml)
10	0.1	5.9	34	10
10	0.5	5.5	34	50
10	1	5	34	100
10	2	4	34	200
10	3	3	34	300

* Retinol stock solution (5mg/ml)

Table 3.4 Composition of the standard (retinol in ethanol) with a total volume of 50 ml

Retinol* (ml)	Absolute ethanol (ml)	Retinol concentration in the sample (μg/ml)
0.1	49.9	10
0.5	49.5	50
1	49	100
2	48	200
3	47	300

* Retinol stock solution 5mg/ml

3.2 Development of a method for extraction of retinol

Although several methods have been reported in the literature to determine retinol concentration in milk, there were no specific methods for retinol in WPI dispersions. Two different experimental procedures were tested in order to obtain the best extraction of retinol. These methods were, (i) direct solvent extraction and (ii) saponification.

3.2.1 Solvent extraction

Two solvent extraction methods from the literature (Gomis *et al.*, 2004, Lloyd *et al.*, 2004) were compared (Table 3.5) and combined in order to extract the retinol in WPI.

Table 3.5 Comparison of methodology used for retinol analysis in milk by Gomis *et al.* (2000) and Lloyd *et al.* (2004)

Procedure	Gomis *et al.* (2000)	Lloyd *et al.* (2004)
Sample preparation	5 ml milk +5 ml ethanol with 0.025% BHT in a 10 ml test tube	5 g of Non-fat dry milk powder dissolved in 30 ml of distilled water.
Sonication to disrupt fat globule membrane	Sonicated for 2 minutes	Sonicated for 5 minutes
Separation	Transferred in to a separatory funnel and 15 ml hexane added. Vortexed for 5 minutes.	Transferred in to a separatory funnel, shaken for 2 minutes with 60 ml hexane.
Extraction	Organic layer transferred and extraction repeated with another 15 ml hexane	The hexane phase was decanted and extraction was repeated twice (2 x 60 ml).
Washing	Both organic layers were combined and washed with 2 portions of 5 ml methanol: water (9:1).	Hexane layers were combined and washed twice with 140 ml aqueous methanol (methanol:water = 8:2).

In the combined method to extract retinol and allow analysis (Figure 3.1), 10 ml from each sample, standard and control was measured into individual separation funnels followed by addition of 15 ml of hexane. Each sample was vortexed for 2 minutes and the top (hexane) layer was decanted into a round bottom flask. Extraction was repeated with another 15 ml aliquot of hexane. The hexane extracts were combined in another separation funnel and washed twice with 5 ml of methanol:water (9:1). The organic upper layer was separated into a round bottom flask and evaporated to dryness using a rotary evaporator (Haake D1, Thermo-Haake Ltd., Karlsruhe, Germany) at 40 °C. The residue was dissolved in 5 ml of methanol, and filtered through 0.45 μm Millipore® filter for HPLC analysis as described in section 3.3.1.

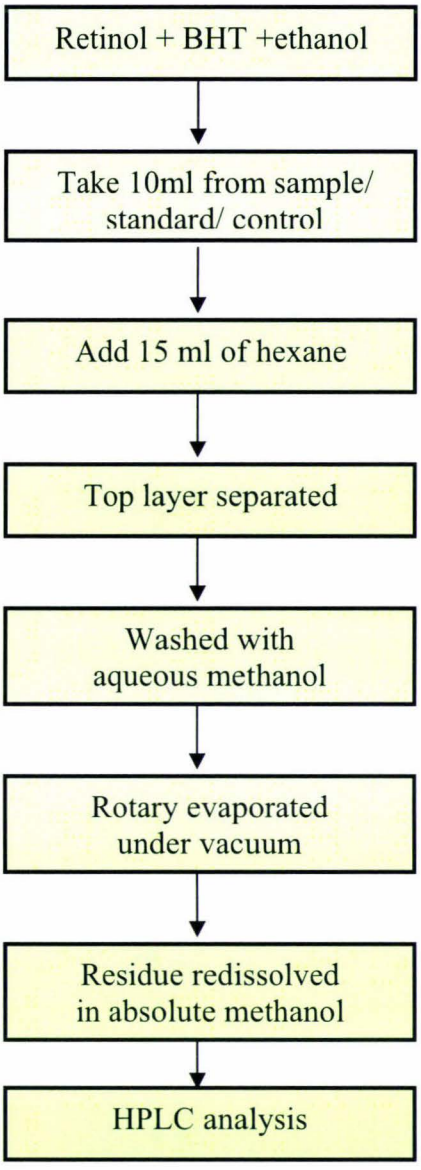

Figure 3.1 Solvent extraction method (Combined from Gomis *et al.* (2000) and Lloyd *et al.* (2004))

The chromatograms were obtained and the areas under the peaks representing retinol were plotted against the known concentrations to obtain the calibration curve. The variations in the quantities of retinol extracted from the different samples, controls and standards using this method resulted in an inconsistent calibration curve. In addition, the WPI appeared to have interfered with the retinol peak, resulting in the

need to subtract the peak area of the WPI in order to obtain the actual retinol peak area.

3.2.2 Saponification

The second extraction procedure tested involved the conversion of retinol esters to retinol (saponification), as described by De Vries *et al.* (2002). Concentrations used by these authors were considerably higher with regards to fortification regulations and the recommended dietary intake (RDI) specifications in Australia and New Zealand. Therefore, retinol standard concentrations needed to be adjusted to comply with the Australia-New Zealand RDI and the fortification regulations. The tolerable upper intake level (UL) of vitamin A was also considered in making this decision. RDI for an adult man over 18 years of age is 900 μg and for an adult women 700 μg per day. The tolerable upper intake level (UL) is 3000 μg per day. Maximum content of vitamin A (including natural and synthetic forms) that is legally allowed in dairy products is 0.625 μg/ml. Considering this level, the new concentrations of samples were set at 6.25, 12.5, 18.75, 25, 31.5 and 37.5 μg/ml. These concentrations, which are higher than RDA, were necessary due to the limited sensitivity of the method that was observed using 0.625 μg/ml as the starting concentration.

A stock solution of retinol acetate was prepared by measuring 27.7 mg of retinol acetate (90% pure) into a 50 ml volumetric flask, and making to volume in the dark to obtain a final concentration of 250 μg/ml. This stock solution was diluted with ethanol to prepare the standards used to obtain a calibration curve (6.25-37.5 μg/ml) (Table 3.6). Samples were prepared with 10% WPI solution and the phosphate buffer (Table 3.7) while the final concentration of WPI was set to be 1% throughout the experiment. Control I was prepared with the phosphate buffer (Table 3.8). Control II was prepared with 10% WPI (4 ml) in phosphate buffer (36 ml). Approximately 50 mg of pyrogallic acid was added to each sample, standard, control I and control II as the antioxidant. All solutions were stored in the dark at 5 °C prior to extraction, followed by the HPLC analysis as described in section 3.3.

Table 3.6 Composition of the standards with a total volume of 40 ml

Retinol acetate* (ml)	Absolute ethanol (ml)	Retinol acetate concentration (μg/ml)
0.5	39.5	6.25
1	39	12.5
1.5	38.5	18.75
2	38	25
2.5	37.5	31.25
3	37	37.5

* Retinol acetate stock solution (500μg/ ml)

- 50 mg of pyrogallic acid was added to each flask

Table 3.7 Composition of the samples with a total volume of 40ml.

10% WPI (ml)	Retinol acetate (ml)	Absolute ethanol (ml)	Phosphate buffer (ml)	Retinol acetate concentration (μg/ml)
4	0.5	4	31.5	6.25
4	1	3.5	31.5	12.5
4	1.5	3	31.5	18.75
4	2	2.5	31.5	25
4	2.5	2	31.5	31.25
4	3	1.5	31.5	37.5

* Retinol acetate stock solution (500 μg/ ml)

- 50 mg of pyrogallic acid was added to each flask.

Table 3.8 Composition of the control I with a total volume of 40 ml.

Retinol acetate* (ml)	Absolute ethanol (ml)	Phosphate buffer (ml)	Retinol acetate concentration (µg/ml)
0.5	4	35.5	6.25
1	3.5	35.5	12.5
1.5	3	35.5	18.75
2	2.5	35.5	25
2.5	2	35.5	31.25
3	1.5	35.5	37.5

* Retinol acetate stock solution (500 µg/ml).

- 50 mg of pyrogallic acid was added to each flask

The analytical procedure was carried out (Figure 3.2) as described by De Vries *et al.* (2002). Briefly, 2ml of each sample was added to 38 ml of absolute ethanol in round bottom flasks covered with tin foil and containing a glass bead to promote even boiling. A portion of (50 mg) of pyrogallic acid was also added to each flask as the antioxidant. Continuous flow of nitrogen (N_2) gas was maintained throughout the experiment. Ten ml of 50% KOH was added into each flask, immediately attached to the reflux condenser. All flasks were refluxed for 45 minutes at 68 °C, and were swirled every 10 minutes to ensure even dispersion of the material.

After this time, the heat was removed and N gas flow was slightly decreased for approximately 5 minutes in order to drain any sample trapped in the reflux condenser. The flasks were then removed from the condensers, stoppered with corks and cooled quickly to the room temperature using a cold-water bath. Once cooled, 10 ml of glacial acetic acid was added into each flask, mixed well, and allowed to return to the room temperature. This cooling step was required as the addition of glacial acetic acid causes an exothermic reaction.

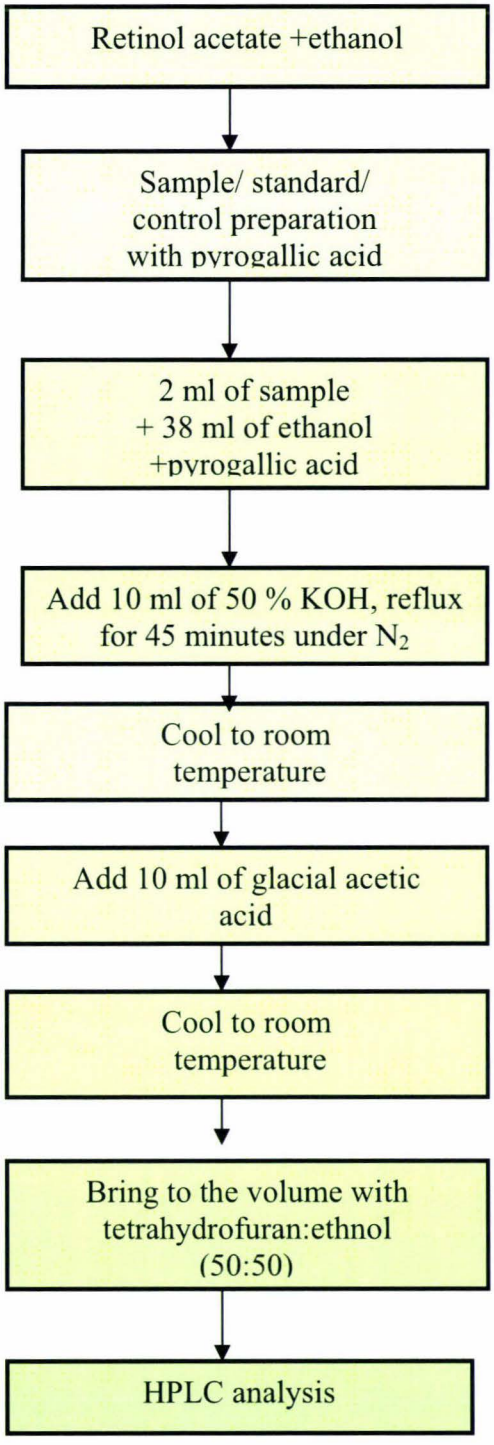

Figure 3.2 Saponification, which converts the retinol acetate into retinol, as detailed in De Vries *et al.* (2002)

The solutions in each flask were transferred using a mixture of tetrahydrofuran (THF) and 95% ethanol (ETOH) (50:50) to 100 ml volumetric flasks. Each flask was well rinsed with the solvent mixture during the transfer process to ensure the entire sample was recovered. Flasks were covered in foil, made up to the volume, then stoppered and inverted 10 times before being stored at 5°C. The final chemical composition of the saponified sample is shown below (Table 3.9).

Table 3.9 Chemical compositions of the solutions at the end of the saponification, with a total volume of 100 ml

Sample (ml)	ETOH (ml)	KOH (ml)	Acetic acid (ml)	THF: Ethanol (ml)	[Retinol]** (μg/ml)		WPI (%)	
					Original	Final	Original	Final
2	38	10	10	40	6.25	0.125	1	0.2
2	38	10	10	40	12.5	0.25	1	0.2
2	38	10	10	40	18.75	0.375	1	0.2
2	38	10	10	40	25	0.5	1	0.2
2	38	10	10	40	31.25	0.625	1	0.2

** Retinol acetate concentration.

Approximately 3 ml of supernatant from each flask was filtered through 0.45 μm filter and subsequently analysed by HPLC as described in section 3.3.

3.3 HPLC Analysis

For all the HPLC analysis, a Waters 2690 HPLC instrument (Alphatec systems Ltd., & Co., Auckland) was used with a Prevail® C18 column (5 μ, 4.6 X 150 mm) equipped with a guard column. The flow rate was set at 1 ml/min with an injection volume of 20 μl and a column temperature of 25 °C. The absorbance was read using a Waters 2487 absorbance detector (Alphatec systems Ltd., & Co., Auckland).

3.3.1 HPLC analysis for solvent extraction

Analysis was carried out with acetonitrile (CH_3CN) and methanol (CH_3OH) as the mobile phase in a 3:1 ratio. The solutions were vacuum degassed and helium sparged. The absorbance was read at 325 nm, which was reported as the most suitable absorbance wavelength for retinol analysis in the literature.

3.3.2 HPLC for saponification

Methanol and Milli-Q water were used as the mobile phase in a ratio of 86:14. The absorbance used to determine retinol levels was 325 nm, which is widely reported in the literature.

3.4 The calibration curve with saponification

The reproduced results provided clear chromatogram peaks with no interference from WPI. Therefore it was concluded that there was no further need of control-II (WPI with no retinol). The sample, control, and standard solutions of 6.25, 12.5, 18.75, 25, 31.25 μg/ml were repeated in triplicates to obtain the calibration curve. For verification for future trials including the light, processing temperature/ time, and storage temperature/time trials the calibration curve run was completed with 12.5, 25, and 37.5 μg/ ml concentration.

3.5 Preparation of samples and controls for the light / storage temperature trial

The samples (S) and controls (C) were prepared in triplicates as shown in Table 3.10. The retinol concentrations used were 12.5, 25 and 37.5 μg/ml. The antioxidant, pyrogallic acid was added (50 mg) to each sample and control vials, which were foil wrapped to protect from light.

Table 3.10 Composition of 40 ml samples (S) /controls (C) used for the light and storage temperature trials

Sample name	Buffer (ml)	WPI (ml)	Ethanol (ml)	Retinol * (ml)	[Retinol]** (μg/ml)
$C_{37.5}$	35.5	0	1.5	3	37.5
$S_{37.5}$	31.5	4	1.5	3	37.5
C_{25}	35.5	0	2.5	2	25
S_{25}	31.5	4	2.5	2	25
$C_{12.5}$	35.5	0	3.5	1	12.5
$S_{12.5}$	31.5	4	3.5	1	12.5

* Retinol acetate stock solution (500 μg/ml).
** Final concentration of retinol acetate.

3.5.1 Effect of WPI on retinol stability in the presence of light

The effect of light on retinol acetate was studied using Phillips® ecotone, 11W cool white fluorescent tube, in a temperature controlled (5°C) incubator (Sanyo 252, Sanyo Electric. Co., Ltd, Japan). The light intensity was measured with a lux meter (Topcon IM-5, Light Lab International, Queensland, Australia) and was ranging between 1200-1350 lx. Samples and controls were prepared in triplicates as described in section 3.5 with concentrations of 12.5, 25, and 37.5 μg/ml of retinol acetate. Aliquot collecting time intervals were dependent on the concentration of the retinol in sample as samples containing higher concentrations of retinol acetate were expected to degrade slower. Time intervals for aliquot collection for sample containing 12.5 μg/ml and 25 μg/ml ranged from 0 to 54 hours and 0 to 74 hours, respectively. At the highest concentration of 37.5 μg/ml, the time intervals ranged from 0 to 96 hours. The processing and analysis of the obtained aliquots were carried out, using saponification as described in section 3.2.2 and HPLC analysis as described in section 3.3.2.

3.5.2 Effect of WPI on retinol stability with regards to the storage temperature

Effect of storage temperature on the degradation of retinol acetate was studied at 40 °C and 5 °C. Samples and controls with concentrations of 12.5, 25, and 37.5μg/ml of retinol acetate were prepared in triplicate as described in section 3.5.

For the 40 °C trial, the samples and the controls were prepared, foil wrapped to protect from light and stored at 40 °C in an incubator (Contherm 1300, Contherm Scientific Ltd., Wellington, New Zealand). The time intervals for samples containing 12.5 μg/ml and 25 μg/ml of retinol acetate ranged from 0 to 19 hours and 0 to 39 hours, respectively. Aliquots were collected starting at 0 and continued up to 72 hours for the sample containing concentration of 37.5 μg/ml retinol acetate. The obtained aliquots were processed immediately as described on section 3.2.2 and analysed according to section 3.3.2.

For the 5 °C storage trial, the samples and the controls were prepared, foil wrapped to protect from light and stored at 5 °C cold storage room. At first, aliquots from the samples with the highest retinol acetate concentration (0.75 μg/ml) were obtained during 0 to 32 hours and continued up to 10 days. A fluctuation of the retinol content was observed in both samples and controls, without a significant degradation, during these time intervals. Therefore, a subsequent set of samples and controls in all three concentrations were used with longer time intervals. Each obtained aliquot was processed immediately as described on section 3.2 .2 and followed by HPLC analysis as described in section 3.3.2.

3.5.3 Processing temperature trial

Pasteurisation (72°C for 15 seconds) and UHT (144°C for 3-4 seconds) were chosen as relevant thermal processing temperatures due to their frequent application in food processing. Samples (S) and controls (C) were prepared in larger quantities (as described below in Table 3.11) as required for circulation through the UHT plant.

Table 3.11 Composition of the samples (S) /controls (C) of 1 L , used for the processing temperature trials

Sample name	Buffer (ml)	WPI (ml)	Ethanol (ml)	Retinol * (ml)	[Retinol]** (μg/ml)
$C_{37.5}$	887.5	0	37.5	75	37.5
$S_{37.5}$	787.5	100	37.5	75	37.5
C_{25}	887.5	0	62.5	50	25
S_{25}	787.5	100	62.5	50	25
$C_{12.5}$	887.5	0	87.5	25	12.5
$S_{12.5}$	787.5	100	87.5	25	12.5

*Retinol acetate stock solution of (500μg/ml)
**Retinol acetate concentration of the sample

Approximately 1.25 g of pyrogallic acid was added to each sample and control, which were foil wrapped to protect from light. Aliquots were obtained before and after the heat treatments (see above). Aliquots were processed as described in section 3.2.2 and were analysed with HPLC according to the section 3.3.2.

CHAPTER 4

DEVELOPMENT OF A METHOD FOR EXTRACTION OF RETINOL

4.1 Introduction

A number of methods to extract retinol from a sample for analysis have been reported in the literature. The two main methods are direct solvent extraction and saponification (Ball, 1999). Solvent extraction has been used widely to extract all-*trans*-retinol while saponification has often been used to extract retinol acetate and retinol palmitate. Solvent extraction involves directly washing the sample using solvents, such as hexane to extract retinol. Saponification is a process that involves alkali treatment, which converts retinol esters to all-*trans*-retinol and extracts the retinol. Both methods have been used for vitamin A quantification in foods. However, no method has been reported to specifically extract retinol from a WPI solution. The aim of this chapter was to carefully analyse the reliability of these two methods to extract retinol from WPI and retinol mixture, and identify the most appropriate method for future experiments.

4.2 Preparation of standard curve

The separation capacity of the HPLC was monitored using retinol standards. These displayed a clear peak at approximately 3.5 minutes. The area under the curve for each peak was plotted against the known concentrations of the standards (Figure 4.2). There was a linear relationship between the known concentration of retinol and the area under the curve for concentrations between 0 to 300 μg/ml.

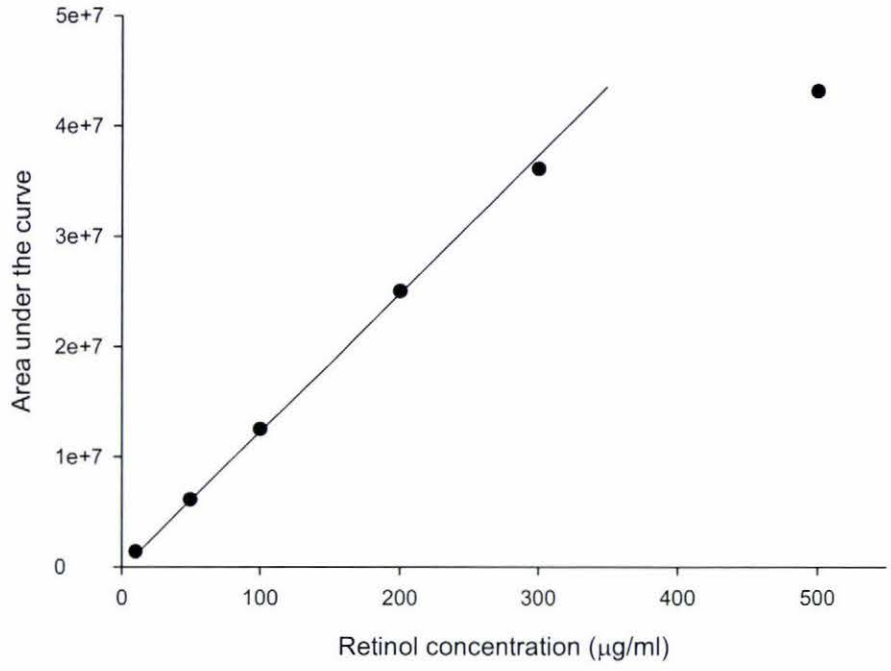

Figure 4.1: The area under the curve for the retinol peak (at 325 nm) for known retinol concentration in HPLC grade ethanol (•). R^2 was 0.977.

The linear relationship observed at these concentrations confirmed that this method could be used to determine unknown retinol concentrations based on peak area. However, the chromatogram for the highest retinol concentration (500 µg/ml) had a flat-topped peak implying that the absorbance at this concentration was beyond that could be measured accurately by the detector. Based on this observation, a concentration range of 10-300 µg/ml was used for future experiments.

4.3 Solvent extraction of retinol from the aqueous phase

Hexane was used to wash and extract all-*trans*-retinol from the sample. Both the hexane and sample were clear and colourless, but since hexane is known to be less dense than water, the top layer containing the hexane and extracted retinol was carefully separated from the lower aqueous layer. Full details of the separation method are given in section 3.2.1 in Chapter 3. The separated hexane layer was further washed with aqueous methanol, which was then evaporated and redissolved in ethanol for HPLC analysis. At 325nm, the extracted retinol samples showed a single

clear peak ~ at 3.5 minutes confirming retinol was present in the extract. However, the extract from the control containing WPI and buffer with no added retinol also had a peak at approximately the same time elution time (Figure 4.2).

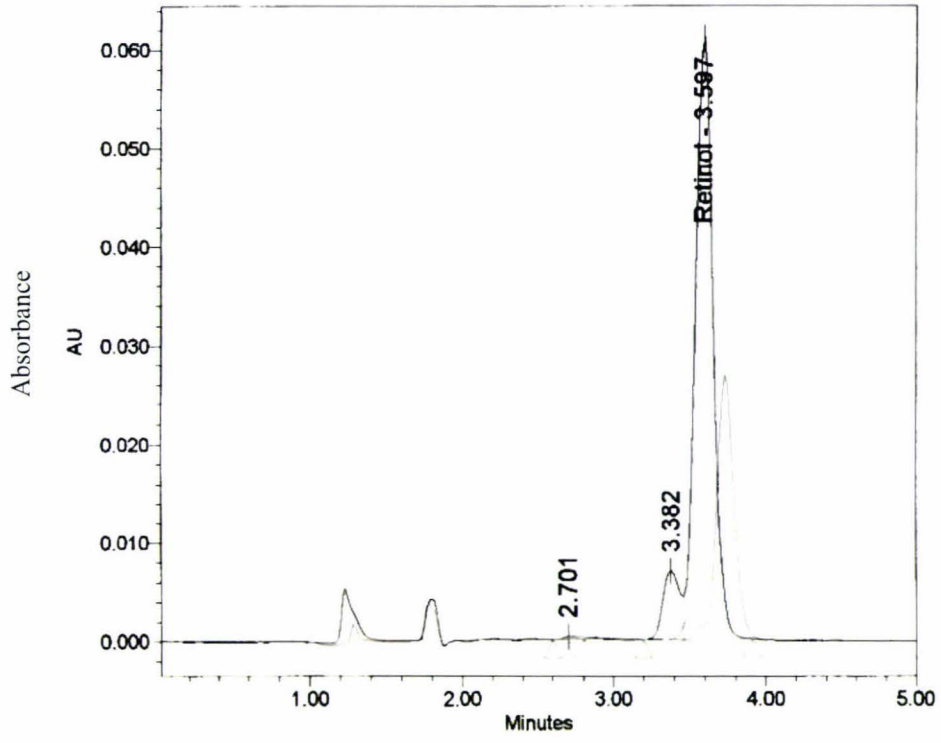

Figure 4.2: Superimposed chromatograms of aqueous-phased all-*trans*-retinol (-) and aqueous-phased WPI ( ) at 325 nm. The gray line, which represent the WPI appear to interfere with the all-*trans*- retinol eluted ~ 3.5 minutes.

This indicates some component from the WPI was also eluting at this time and absorbed at the same wavelength. Therefore, to determine the actual area that corresponded to the added retinol, the area under the curve for that peak in the sample containing WPI was subtracted from the peak area of all samples, which contained WPI. The known concentrations of the samples and the peaks were then used to obtain the calibration curve (Figure 4.3).

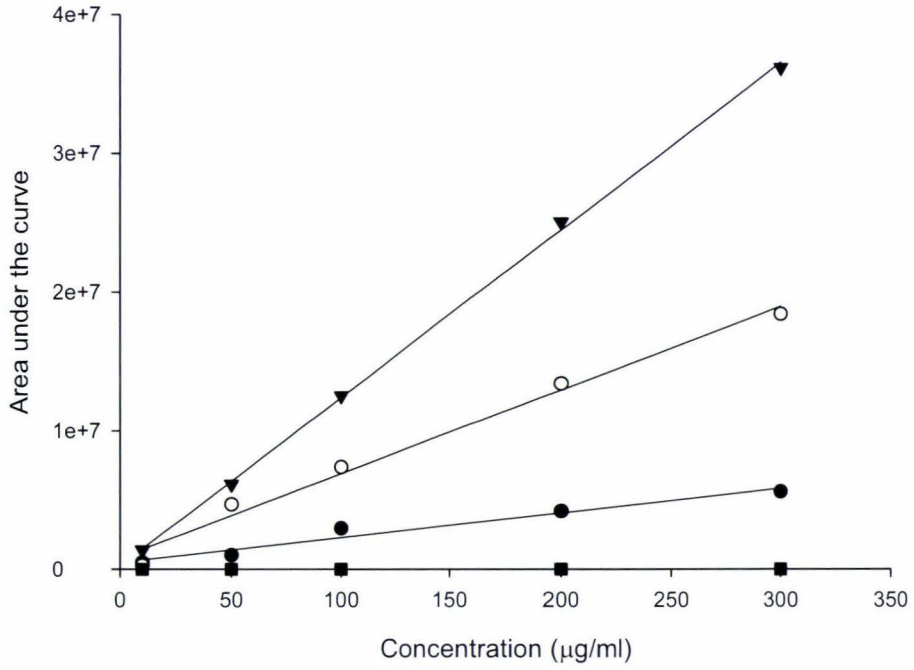

Figure 4.3: Retinol extracted using direct solvent extraction as area under the peak against the known retinol concentrations of retinol in ethanol (▼), retinol in buffer (●), retinol with WPI (○) and WPI in buffer (■) with R^2 values of 0.9990, 0.9609 and 0.9863 respectively.

Retinol is readily soluble in solvents, such as ethanol and hexane (Ball, 1999). The standard of retinol in ethanol was used to identify the amount of retinol, which should have been present in the samples and controls if 100% of the added retinol had been extracted. After the normalisation of the data, the percentage of recovery showed a significant variation between the different solutions (Table 4.1). Overall, samples (containing WPI) showed slightly better recovery than controls (no WPI), but there were no identifiable trends in the extent of recovery.

Table 4.1: Percentages of retinol recovered from samples, using solvent extraction.

Added retinol (μg/ml)	Retinol extracted from control (retinol in buffer) (%)	Retinol extracted from sample (retinol and WPI in buffer) (%)
10	34.5	20.5
50	16.5	76.5
100	23.5	58.5
200	17.5	53.5
300	15.5	51.0

The retinol recovery varied from 15 to 76%, regardless of the initial retinol concentration. This variation may have resulted from the inability of the hexane to access the retinol in an aqueous environment, but clearly indicates that this method is not reliable in this system and thus cannot be used for further experiments.

4.4 Extraction of retinol from the aqueous phase using saponification

The saponification method was tested using standards (retinol in ethanol), controls (retinol and buffer) and samples (retinol and WPI in buffer). The retinol concentrations were reduced to more accurately reflect the retinol levels likely to be encountered in food products, and ranged from 0.625 to 37.25 μg/ml, and a different form of vitamin A (retinol acetate) was used. Full details of this method are given in section 3.2.2. During the saponification, an aliquot of the standard, control or sample was subjected to an alkaline treatment using KOH, which converts retinol acetate to retinol. The KOH changed the colour of the sample from pale yellow to black. During refluxing, the sample colour remained unchanged. The addition of acetic acid caused an exothermic reaction, so the samples were immediately sealed and cooled to room temperature before the supernatant was taken for HPLC analysis. The chromatograms of the supernatants showed a single peak at ~12.2 minutes (Figure 4.4). The peak areas were plotted against their known concentrations to obtain the calibration curve (Figure 4.5).

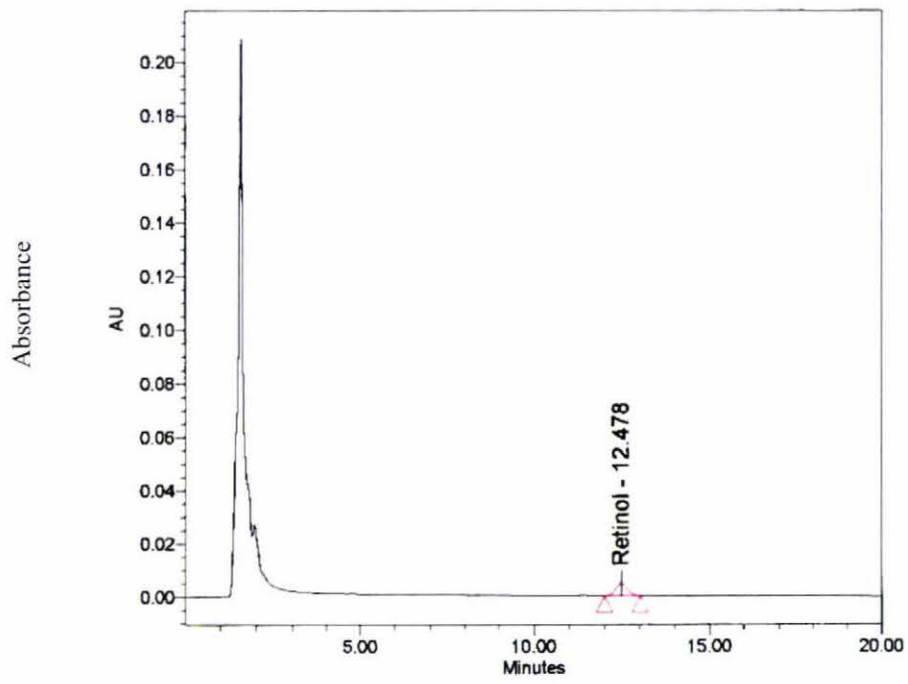

Figure 4.4: A chromatogram from the saponification showing a clear single peak at ~ 12.2 minutes.

Figure 4.5 showed a linear relationship between the retinol concentration and the area under the curve for all three solutions. The lowest retinol acetate concentration was 0.625 μg/ml, which corresponded to a concentration of 0.125 μg/ml injected into the HPLC after all the dilutions. Solutions containing this amount of retinol acetate had no peaks in the chromatograms, indicating the inability of the method to detect retinol at such a concentration, even though De Vries *et al.* (2002) stated that this method was suitable for the determination of retinol from 0.15 μg/g. The amount of retinol extracted from the controls and samples was very similar to that extracted from the standard, but the amount of retinol extracted at the highest concentrations (31.25 and 37.5 μg/ml) appeared to be slightly higher than expected. These data points were obtained in a separate run from the other samples, controls and standards, and this slight variation may be due to fluctuations in the HPLC equipment.

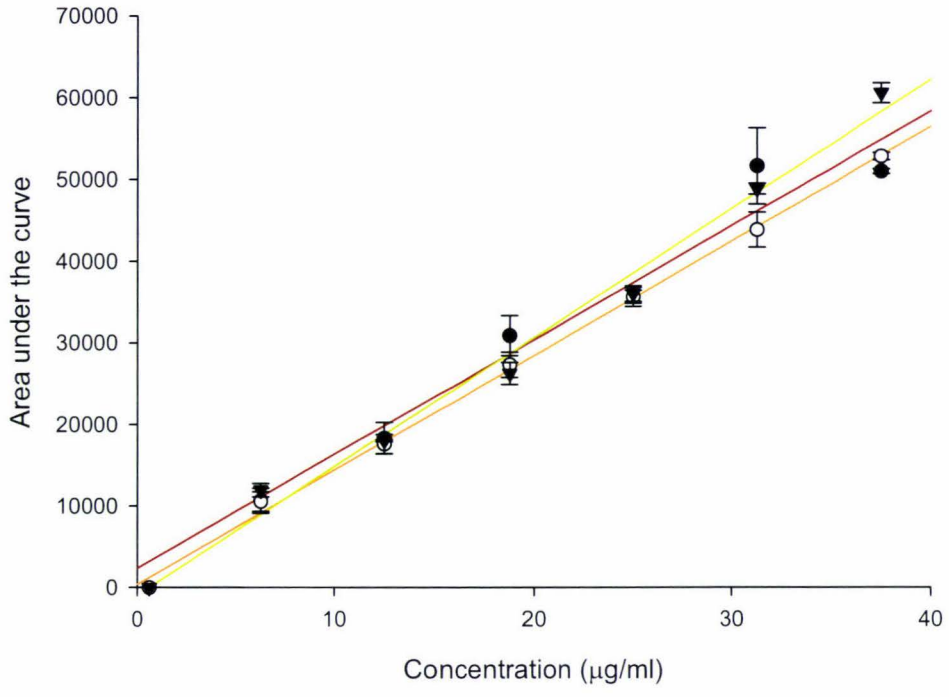

Figure 4.5: Retinol extracted using saponification technique as area under the peak against the known retinol concentrations of retinol in ethanol (●), retinol in buffer (○) and retinol in WPI (▼), with R^2 values of 0.9596, 0.9980 and 0.9898, respectively.

The percentage recovery of the retinol from the samples and controls was calculated and is shown in Table 4.2. These were consistently close to 100%, and are much higher than the results obtained from the solvent extraction technique (Table 4.1). The percentages in excess of one hundred percent are likely to be due to small fluctuations in the HPLC equipment as the replicate results were obtained in different runs.

Table 4.2 Percentages of retinol extracted from samples, using the saponification.

Added retinol (μg/ml)	Retinol extracted from control (retinol in buffer) (%)	Retinol extracted from sample (retinol and WPI in buffer) (%)
0.625	0	0
6.25	98	112
12.5	96	98
18.75	88	84
25	100	101
31.25	84	94
37.5	103	118

4.5 Discussion

The poor recovery level of the direct solvent extraction may be attributed to the fact that the method is not specifically designed for extracting retinol from protein solutions. Since retinol is a fat-soluble vitamin, the extraction method may be suitable for extracting retinol when it is associated with the lipid-phase, but may not work when trying to extract retinol from an aqueous system, which does not include any fat. The interference caused by the WPI during HPLC analysis would also add to the difficulties in using this method. The inconsistencies in the extraction percentages gave a clear indication of the unsuitability of this method for studying the interactions between retinol and WPI.

In contrast, saponification is a universally-accepted technique for extracting vitamin A from almost any type of composite food sample (Ball, 1999). This method delivered reliable chromatographs without any interference from WPI. A single peak was observed at ~12.2 minutes, slightly longer than the retention time reported by De Vries *et al.* (2002) of ~ 9 minutes. This can be explained by the slightly different column used in this experiment (C18, 5 μ, 150 mm x 4.6 mm) compared to that used by De Vries (C18, 10μ, 250 mm x 4.6mm). The percentage of retinol extracted from

the samples and controls was consistently close to 100% of the added retinol, a dramatic improvement on the results obtained using solvent extraction.

The major differences between the two methods used may reflect not only the differences in processing, but also in the form of vitamin A added to the samples. For saponification, the more stable retinol acetate, (as reported by Woolard *et al.* (2003)) was used, while the less stable all-*trans*-retinol was used for solvent extraction. This is reflected by the stability of the vitamin A stock solutions, with retinol acetate stock solutions stable for two weeks (De Vries *et al.*, 2002).

Saponification involved heating the sample with alkali treatment whereas the solvent extraction involved solvent washes of the sample at the room temperature. Although vitamins are generally heat labile, the boiling point of all-*trans*-retinol is 137 °C (Weast *et al.*, 1982). This suggests heating the sample during saponification is unlikely to result in any retinol losses due to the very high boiling point of retinol. Also, vitamin A is relatively resistant towards alkali (Woolard *et al.*, 2003), so the alkali treatment is not likely to result in retinol losses. The other advantage of saponification is the use of N_2 atmosphere to minimise the oxidative stress on retinol, which is considered essential to a successful analysis (Woolard *et al.*, 2003). However, this procedure is extremely time consuming and requires large amounts of solvents.

In conclusion, saponification, despite its lengthy process, appears to be a reliable method for the extraction of retinol acetate from aqueous systems and hence was the method of choice for all further experiments.

CHAPTER 5

EFFECT OF WPI ON THE STABILITY OF RETINOL IN THE PRESENCE OF LIGHT

5.1 Introduction

The effect of light on the stability of vitamin A has been extensively reported in the literature. The source, wavelength, intensity and the length of exposure to light (Sattar *et al.,* 1977; Gaylord *et al.*, 1986; Murphy *et al.*, 1988; Fellman *et al.*, 1991) result in photo-isomerization of vitamin A, which leads to photo-oxidation. Photo-isomerization and photo-oxidation reduce the vitamin A activity (Murphy *et al.,* 1988). The effect of physical barriers, such as wavelength filters, labels and the use of biochemical barriers, such as emulsion/carrier systems (Zahar *et al.*, 1992) have been used to prevent light-induced degradation of vitamin A (refer to chapter 2).

Vitamin A has been shown to bind to a number of whey proteins, including β-Lg and BSA (Futtreman *et al.*, 1972; Puyol *et al.*, 1991; Considine *et al.*, 2005). The main binding site for retinol (the most common form of vitamin A) in β-Lg is located internally in the folded protein (Kontopidis *et al.*, 2002) although there are external binding sites (Monaco *et al.*, 1987). This indicates the possible functionality of β-Lg as a carrier or a mediator (Swaisgood *et al.*, 1998) for retinol. Therefore, it would be interesting to gain an understanding of the possible functionality of WPI as a carrier molecule with regards to stabilisation of retinol in an aqueous environment. The aim of this chapter was to study whether WPI had any influence on the stability of vitamin A in an aqueous environment, when exposed to light.

5.2 Degradation of retinol in the presence of light

The effect of WPI on vitamin A in the presence of light was investigated using retinol acetate concentrations of 12.5, 25 and 37.5 μg/ml. All samples and controls were stored at 5 °C to prevent thermal degradation. The retinol acetate concentrations in

samples were monitored over a period of 0 to 96 hours, where the time intervals were selected based on the initial concentration. Higher retinol acetate concentrations were expected to remain in the sample for a longer period of time and hence longer time intervals were used to obtain samples for analysis. Statistical analysis of the results was performed using a general linear model procedure with a log transformation, developed by SAS -V8 institute inc. (Cary, NC). The model included the storage time, retinol acetate concentration and the difference between the degradation trends of the sample and control.

5.2.1 Effect of light on the degradation of 12.5 μg/ml of retinol acetate

Retinol acetate degraded over a very short period of time (Figure 5.1), in the sample and control containing initial retinol acetate concentrations of 12.5 μg/ml, with a complete degradation of retinol acetate occurring within 48 hours of light exposure. In general, rapid degradation of retinol acetate occurred within the first 9 hours (nearly 40 % loss) and then continued more slowly between 9 to 49 hours.

The extent of degradation appeared similar in both the control and the sample (with added WPI) from 9 to 31 hours. Between 9 and 31 hours, only additional 15 % of retinol acetate was lost. The antioxidant (pyrogallic acid) was added in small amounts (1.25 mg/ml) to both solutions during the preparation, which could be responsible for this stabilising effect. After 48 hours, the complete loss of all retinol acetate was observed in the control, while the sample still retained 30 % of the initial retinol acetate content. This indicates a potential but limited protective effect of WPI on retinol acetate during exposure to light for extended periods.

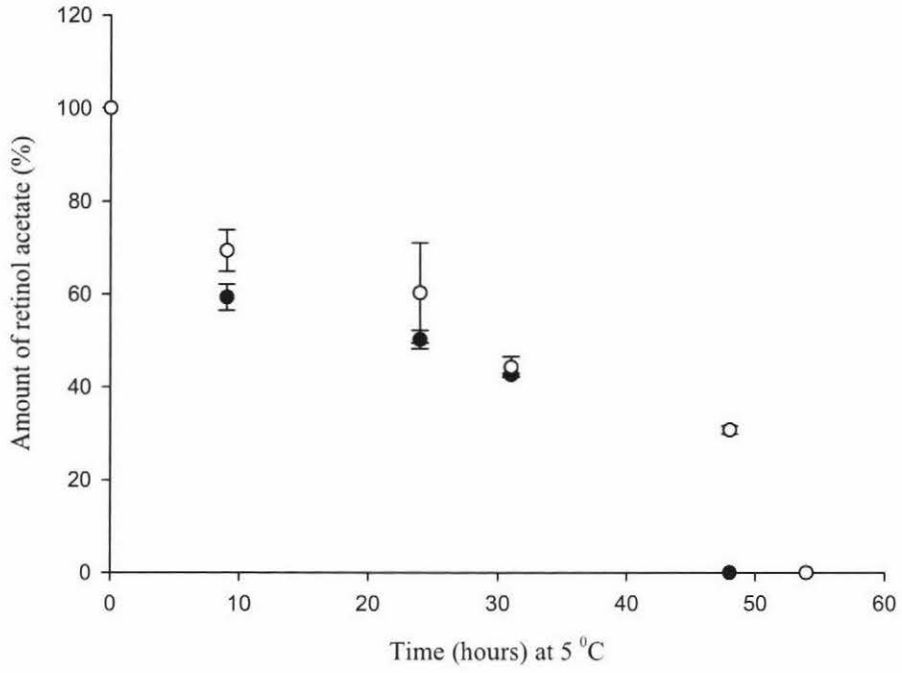

Figure 5.1: Degradation of retinol acetate (12.5 μg/ml) as a percentage, in the presence of light. Percentages of retinol in control (●) that contain retinol in buffer, and samples (○) that contain retinol in buffer with WPI, at each time interval are shown here.

The degradation trends of the sample and the control were compared and statistical analysis was carried out using SAS (V 8.2). The two variables in the statistical model, the "time" and the "intercept" (the concentration at the beginning) were highly significant. ($p < 0.0001$). Although the sample appeared to retain more retinol acetate at certain times along the degradation trend, the difference between slopes (using 95 % confidence intervals) was 0.10 ($p > 0.05$), indicating this difference was not statistically significant. This suggests that WPI did not have a statistically significant protective effect on retinol acetate at this concentration

5.2.2 Effect of light on the degradation of 25 μg/ml of retinol acetate

The degradation trends of the retinol acetate in the samples and controls that contain 25 μg/ml of initial concentration are shown in Figure 5.2. Rapid loss of retinol acetate occurred during the first 9 hours of light exposure, similar to the observations made at the lower retinol acetate concentration. During this period, ~ 40 % of retinol acetate was lost from the control and ~ 35% from the sample. There was a reduced rate of degradation occurred during the subsequent 48 hours, with no apparent difference in retinol acetate concentration between the control and the sample.

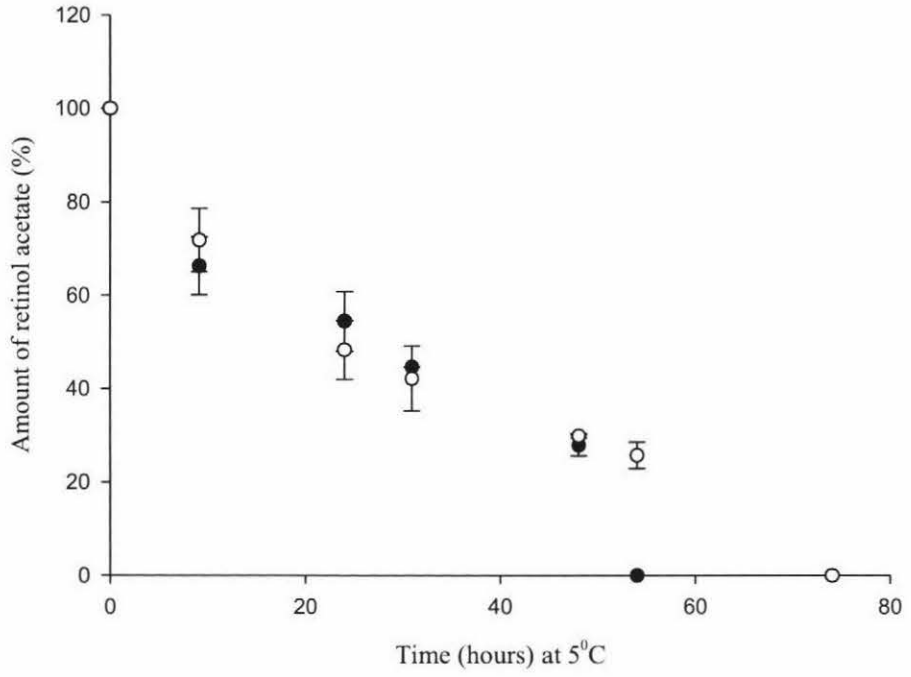

Figure 5.2 Degradation of retinol acetate (as a percentage) due to light in control (●) and sample (○) containing initial retinol acetate concentration of 25 μg/ml.

Between 48 and 54 hours, all remaining retinol acetate was lost in the control while the sample retained ~ 25 % of the initial retinol acetate, suggesting WPI may have been providing a small protective effect. However, even in the samples containing WPI, total retinol acetate loss occurred by 74 hours.

Statistical analysis was carried out using SAS (V8.2) to compare the differences between the rate of retinol degradation in the control and the sample. This indicated

that the time and the concentration significantly predict the degradation rate (both $p < 0.0001$). However, the difference between the slopes for the sample and control had a p value of >0.05, indicating that the potential protective effect of WPI is not statistically significant.

5.2.2 Effect of light on the degradation of 37.5 μg/ml of retinol acetate

The degradation trend of the retinol acetate in the sample and control containing an initial concentration of 37.5 μg/ml is shown in Figure 5.3.

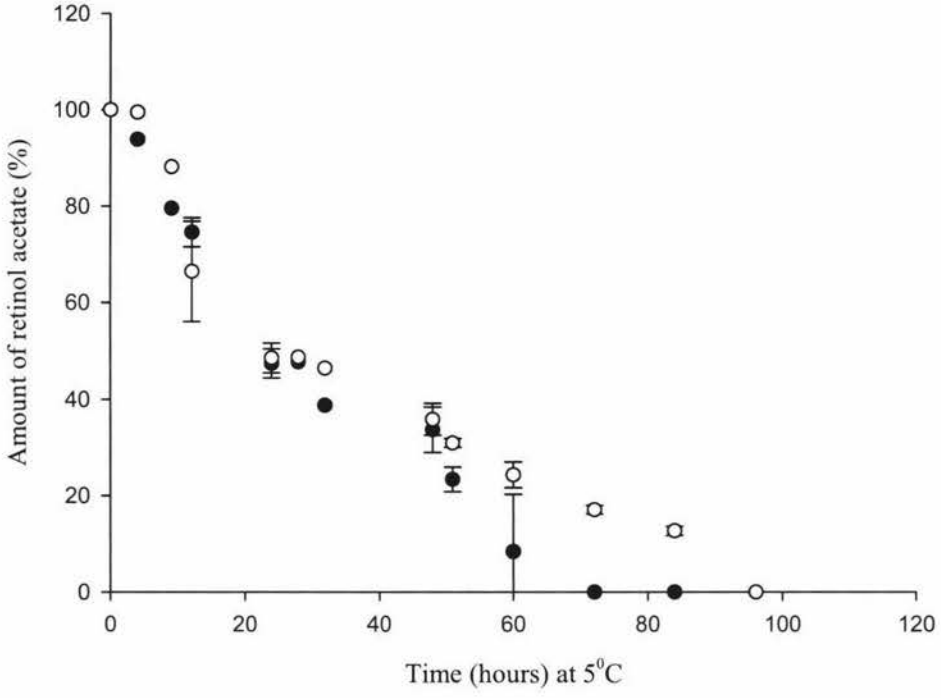

Figure 5.3: Degradation of retinol acetate due to the light exposure, in the control (●) and sample (○) containing initial retinol acetate concentration of 37.5 μg/ml.

Although the degradation trend gives the impression of a rapid loss, the percentage loss that occurred within the first 9 hours was relatively small compared to the previous retinol acetate concentrations (12.5 and 25 μg/ml). After 9 hours of the light exposure, the sample with WPI had lost ~ 12 % of the retinol acetate, compared to the ~ 21 % loss in the control. Both the control and sample showed a high level of retinol

acetate degradation over the first 24-hour period of light exposure (~50 %), regardless of the presence of WPI. During the following 24 hours, a reduced level of degradation was observed and the sample with WPI seemed to retain more retinol acetate compared to the control.

Interestingly, a difference between the degradation trends of the sample and control started to appear after 48 hours. Rapid degradation of retinol acetate occurred in the control while much slower degradation was observed in the sample. By 72 hours, 100 % of retinol acetate had been lost from the control while the sample retained approximately 17 % of the initial retinol acetate content. After 84 hours of light exposure, the sample still retained 12 % of the initial retinol acetate content, although total loss occurred by 96 hours.

The statistical significance was analysed using SAS (V8.2) and once again the time and initial retinol acetate concentration were statistically significant ($p < 0.0001$). This time, the difference between the two trends was highly significant, with a p value of 0.0017. This suggests that WPI has a significant effect on stabilising retinol acetate exposed to light at higher initial concentrations (i.e. 37.5 μg/ml).

5.3 Discussion

Exposure to light resulted in the loss of retinol acetate in all samples and controls. Bertholomew *et al.* (1990) studied the effect of light on the stability of vitamin A in Durafax® or butter oil based emulsions, and similar degradation trends were observed with 50 to 80% loss of vitamin A within 32 hours of light exposure. These researchers found nearly 30 % of vitamin A was lost over the first 8 hours of light exposure, in both types of emulsions. This loss had occurred with the use of a lower retinol concentration (~1.26 μg/ml) and higher light intensity (4300 lx) than that used in the current experiment (1350 lx). Although this experiment was not focused on the effect of light intensity, it has been established that vitamin A degradation is proportional to light intensity (Gaylord *et al.*, 1986) and therefore should be considered when comparing any results.

Overall the samples containing the 3 different initial concentrations of retinol acetate displayed similar degradation trends. There were rapid losses during the initial period of the light exposure, which was followed by a slower rate of degradation and subsequent total loss (Figure 5.4). This suggests that there was a delayed response in the protective effect of the WPI for the retinol acetate, and that protective effect was limited.

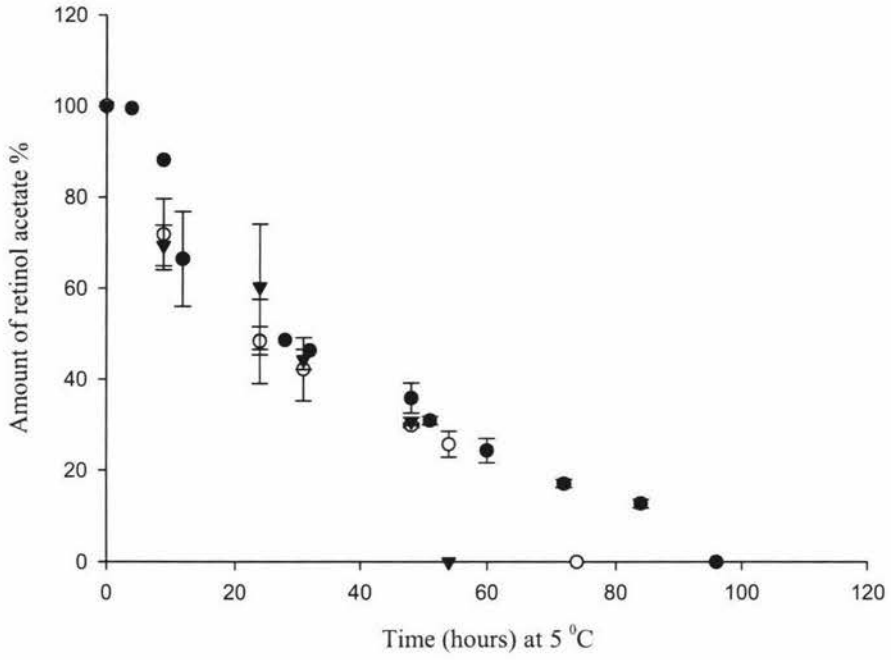

Figure 5.4: Degradation of retinol acetate due to light exposure, in samples with initial retinol concentrations of 37.5 (•), 25 (○) and 12.5 (▼) μg/ml.

The rapid losses observed during the first 24 hours may have been due to the limited binding of retinol acetate to WPI during this period, exposing the vitamin to the risk of photo-oxidation. Schofield (1999) who studied the binding of retinol acetate to WPI stated the binding was faster in the absence of light, but still did occur in the presence of light. If the light exposure was slowing the rate of binding between the retinol acetate and the WPI, it would take longer for any protective effect that may come from such binding to be seen. Then as the level of binding increases, the retinol acetate stability may also increase, slowing the observed rate of degradation. The

amount of degradation was significantly reduced in all three concentrations during the second 24-hours period, when only ~15 % of the retinol acetate was lost. This suggests that a unique factor that was present in the sample may have influenced the vitamin A stability. The unique factor that was present in the sample (but not in the control) was WPI. Schofield (1999) studied the binding of retinol acetate to WPI in buffers and reported that retinol acetate could bind to WPI, and that the WPI had a stabilising effect on the vitamin when the sample was exposed to natural light. The results of the current experiment (which also used retinol acetate) also confirm a small protective effect of WPI on retinol acetate with respect to light and the effects are more evident at higher retinol acetate concentrations (> 25 μg/ml).

Vitamin A is an important nutrient in the human diet and the possibility of using WPI to incorporate vitamin A to food system could be evaluated by interpreting these results in terms of RDA. The amount of retinol retained in each sample as a percentage of RDA is presented in Table 5.1. All the controls had no retinol remaining after the length of light exposure mentioned in this table.

Table 5.1 Amount of retinol left after maximum light exposure in samples as a percentage of RDA (based on a standard beverage serve of 250 ml)

Initial retinol acetate concentration (μg/ml)	Light exposure (Hours)	Percentage of RDA in single serve (%)
12.5	48	2.07
25	54	3.33
37.5	84	22.66

In order to have ~ 23% of the RDA remaining in a 250 ml serving after 84 hours, the fortified product must have an initial retinol acetate concentration of 37.5 μg/ml. This level is approximately 60 times the amount that fortification regulations for vitamin A allow in dairy products in New Zealand. This clearly demonstrates the need for extremely high initial concentrations in order to stabilise a significant amount of retinol acetate in this system and it would be necessary to considerably exceed the current fortification level. In addition, the length of time that the retinol was present in the system at a significant concentration (84 hours) is very low when considering the normal expected shelf life of a food product.

However, it is worth noting that this limited protection by WPI was achieved under the most extreme light degradation conditions. Samples were in clear glassware, directly exposed to light with an intensity of 1350 lx, and background light absorption was minimised using light-reflecting foil. This means that WPI was the sole protection that retinol in the sample possess. It may be possible to significantly improve the vitamin A stability by reducing the severity of these conditions. Effective use of packaging, UV absorbers, wavelength filters and labels had been reported in the literature (Section 2.5.1). Thus a combination of techniques (such as the use of a label that covers considerable area of the container) along with the inclusion of WPI may be worth investigating to maximise retinol acetate retention.

In conclusion, WPI appears to have a small protective effect on retinol acetate when exposed to light. This effect is more noticeable when the retinol acetate is added at higher concentrations and it takes some time to show differentiation between the samples with and without WPI.

CHAPTER 6

EFFECT OF WPI ON THE STABILITY OF RETINOL AT VARIOUS PROCESSING TEMPERATURES

6.1 Introduction

The effect of processing temperature on the stability of retinol has been studied previously (Woolard *et al.*, 1986; Panfelli *et al.*, 1998). The existing data suggests that the thermal stress results in conversion of the predominant all-*trans*- retinol isomers to *cis*-isomers (Woolard *et al.*, 1986) which results in reduced vitamin A activity (Panfelli *et al.*, 1998). High processing temperature also causes thermal denaturation of milk proteins (Jelen *et al.*, 1995). This effect depends on the time and temperature combination that the milk is subjected to, during processing. Pasteurisation at 72 °C for 20 seconds can result in denaturation of about 7 % of the whey protein in milk while UHT treatment at 140 °C for 4-8 seconds may result in 50 to 75 % of whey protein being denatured. Literature indicates that heat-induced conformational changes occur in WPI (Zhu *et al.*, 1994), although some of these changes are reversible. These changes in whey proteins could influence their ability to bind to retinol. Presence of all-*trans*-retinol protected β-Lg from denaturation at temperatures close to 70 °C (Considine *et al.*, 2005).

The aim of this chapter is to gain an understanding of the possible functionality of WPI as a carrier molecule in order to protect vitamin A from degradation caused by heat treatments.

6.2 Effect of WPI on retinol during pasteurisation

Samples with WPI and controls with no WPI were pasteurised at 72 °C for 15 seconds. Sample and control pH values were ranged from 6.9 to 7.2. The amounts of retinol acetate found in the samples and controls after the heat treatment are presented below (Table 6.1).

Table 6.1 Percentages of retinol acetate in samples and controls remaining after pasteurisation.

	Initial concentration of retinol acetate (μg/ml)	Retinol acetate remaining after pasteurisation	
		Absolute amount (μg/ml)	Percentage (%)
Control (No WPI)	12.5	0	0
	25	0	0
	37.5	0	0
Sample (with 1%WPI)	12.5	11.76	94
	25	21.19	84
	37.5	18.57	49

The controls clearly demonstrate that retinol acetate was not stable in the aqueous environment during pasteurisation in the absence of WPI. No retinol acetate was left in any controls after the pasteurisation treatment, regardless of the initial retinol concentration. Therefore, retinol acetate by itself does not appear to be stable in an aqueous environment at pasteurisation temperatures. The complete loss of the retinol acetate also indicated the inability of the antioxidant (pyrogallic acid), added during the preparation of the solutions, to protect retinol acetate under these conditions. In contrast, all samples that contained WPI retained at least some retinol acetate after the pasteurisation treatment. The sample with the lowest initial retinol acetate concentration (12.5 μg/ml) retained 94 %, while the sample with the highest initial retinol acetate retained only 49 %. At the initial concentration of 25 μg/ml, approximately 84 % of retinol acetate was retained after pasteurisation.

The results appear to show an inverse relationship between the initial retinol concentration and the percentage remaining after pasteurisation. This suggests that retinol was more stable in the sample with a low initial concentration compared to the sample with a higher initial concentration. This also implies the stability of retinol was not dependent on the initial retinol concentration. This suggests that a factor other than the retinol acetate concentration determines the amount of retinol acetate

left in the sample after pasteurisation and that this factor was constant in all the samples but was not present in the controls. The concentration of the WPI was consistent at 1% in all the samples. This suggests that the amount of WPI may have been the key factor in the stability of the retinol in samples.

Most of the whey proteins are relatively stable at temperatures such as those used in pasteurisation. The thermal stabilities of individual whey proteins in a simple aqueous system were found to be as 63 °C for α-La, 65 °C for BSA and 74 °C for β-Lg (de Wit *et al.*, 1983). It is widely accepted that β-Lg has specific binding sites for retinol. The binding of retinol to β-Lg may protect the retinol from heat-induced degradation. Interestingly, the binding of retinol to β-Lg, also increases the thermal stability of the protein (Considine *et al.*, 2005).

The statistical analysis of the data was carried out using analysis of variance (ANOVA) produced by SAS (V8) Institute Inc. (Cary, NC). The two-way analysis for the control and sample was highly significant ($p< 0.0001$), indicating the presence of WPI had a significant effect on retinol stability. The statistical significance of the initial retinol acetate concentration of samples was also tested and found to be highly significant ($p< 0.0001$).

6.3 Effect of WPI on retinol during UHT

The samples with WPI and controls with no WPI were subjected to UHT treatment at 144 °C for 4 seconds. The amount of retinol acetate in the controls and samples was determined before and after heat treatments. There was no retinol acetate left in either the controls or samples regardless of the initial concentration. These results showed that the antioxidant was unable to stabilise retinol acetate under these processing conditions used.

The total loss of retinol acetate from all the samples may be explained by the protein denaturation that is likely to have occurred due to the extremely high temperature treatment. The reversibility of the conformational changes in whey proteins decreases with increasing temperatures. For example, temperatures of 80-90 °C resulted in

partial unfolding of β-Lg (Reddy *et al.*, 1994) and subsequent denaturation. This suggests that WPI is not suitable to use as a carrier molecule for products processed using UHT treatment.

The stability of WPI and retinol is strongly pH dependent. Retinol is more stable in alkali conditions (Ball, 1999) and less stable at pH values less than 4.5 (Panfili *et al.*, 1998). In contrast whey proteins are extremely stable at pH values below 3.9 while heating at close to pH 6.8 results in the formation of aggregation (Jelen *et al.*, 1995). The samples and controls used in these experiments had pH values ranging from 6.8-7.4, a range where whey proteins are particularly sensitive to denaturation, potentially limiting their ability to protect the retinol acetate from degradation.

6.4 Discussion

It is possible that the constant amount of 1 % WPI is a critical factor in determining the amount of retinol acetate protected, due to its ability to bind to retinol acetate (Schofield, 1999). Although the stoichiometry of the binding of retinol to WPI as a complex mixture is not known, the stoichiometry of the binding of retinol to β-Lg (the most abundant whey protein) is known to be 1:1 (Futturman *et al.*, 1972, Dufour *et al.*, 1994) and so a constant amount of retinol should have been bound to the β-Lg in the WPI.

The amount of WPI was set at 1 % (0.01 g/ml), and β-Lg comprises approximately 50 % of the protein in WPI. Based on this, the β-Lg concentrations in each sample should be ~ 5×10^{-3} μg/ml or ~2.688×10^{-7} mols, while retinol acetate would provide 3.8051×10^{-8} to 1.1413×10^{-7} mols (refer to the appendix for calculations). Given the 1:1 stoichiometry, one would expect up to 2.688×10^{-7} moles of retinol could become bound to the β-Lg and be protected from degradation. At the lowest (12.5 μg/ml) retinol concentration, 3.73×10^{-8} mol of retinol was left in the sample after pasteurisation, while at 25 μg/ml and 37.5μg/ml, 4.85×10^{-8} and 3.531×10^{-8} mols respectively were left. The amounts of retinol remaining in all the samples are lower

than the amount that could bind to the β-Lg, suggesting that not all the β-Lg was utilised for retinol binding.

It is possible that β-Lg in WPI may have a lower affinity for retinol than native β-Lg in milk. Jang *et al.* (1990) reported that β-Lg obtained by bioselective adsorption had a higher affinity and binding capacity to retinol compared to the β-Lg in WPI. This could mean that the data reflect a saturation effect of retinol β-Lg in WPI. The effect of pH on retinol binding has been reported to be reduced at low pH value such as 5.1, with high affinity observed at pH 7 (Wang *et al.*, 1993). The samples had pH values ranging from 6.8 to 7.4 and retinol binding is therefore unlikely to have been affected by pH.

The results in this chapter may also be used to explain the high stability of retinol in the thermally processed milk that has been reported in the literature. Ford *et al.* (1969) and Le Magure *et al.* (1986) stated that no significant loss of vitamin A occur in pasteurised milk. This stability may be attributed to the other components (such as caseins, milk fat, etc.,) that are present in milk other than the whey proteins. Any of these components may form complexes with retinol (either directly or indirectly) and thereby stabilise it. The results in this thesis demonstrate the ability of WPI to stabilise retinol in an aqueous environment, validating the possible protective effect of WPI on retinol with regards to temperature. Relative stability of retinol in milk at pasteurisation temperature may be at least partially attributed to the milk proteins as opposed to the fat content (Lau *et al.*, 1986). However, at UHT treatment temperatures where high protein denaturation occurs the fat content may be responsible for the reported retention of retinol in milk.

In conclusion, WPI appeared to have a protective effect on retinol acetate during pasteurisation at 72 °C for 15 seconds. However, WPI did not show a significant protective effect on retinol acetate during UHT treatment at 144 °C for 4 seconds, possibly due to the denaturation of the proteins.

CHAPTER 7

EFFECT OF WPI ON THE STABILITY OF RETINOL AT DIFFERENT STORAGE TEMPERATURES

7.1 Introduction

It is well known that vitamin A degrades over time, and the storage temperature appears to be a key factor in determining the rate at which this occurs. Auto-oxidation and isomerisation induced by thermal stress have been attributed to this degradation. The rate of vitamin A degradation is initially rapid but gradually becomes slower, and eventually results in a complete loss of the vitamin (Woolard *et al.*, 1983). In order to control such degradation, antioxidants, packaging and specific vitamin A carrier systems have been used (refer to the literature review section 2.5). Although no particular study has focused on the effect of WPI as a vitamin A carrier, the possibility of using WPI as vitamin A carrier molecule has been suggested (Schofield, 1999). Therefore, it is desirable to gain an understanding of the possible use of WPI to improve retinol stability at different storage temperatures.

Retinol appears to be relatively stable at low temperatures (below room temperature) if the system is maintained free of oxygen and short-wavelength light (Woolard *et al.*, 2003). In contrast, during storage at temperatures above the ambient, retinol degradation appears to be inevitable (deBore *et al.*, 1983). The aim of this chapter is to study the effect of WPI on retinol with regards to storage at 5 °C and at 40 °C. Stability at 5 °C is obviously important, with many food products being stored at refrigeration. Stability at 40 °C was assessed to reflect the temperatures, which certain food products (e.g. milk powder) are likely to be subjected during transportations and storage, particularly in countries with tropical climates.

7.2 Storage at 5 °C

7.2.1 Preliminary study

A preliminary study was carried out to observe the stability of retinol acetate at 5 °C using an initial retinol acetate concentration of 37.5 μg/ml. All samples and controls used to study the effect of WPI on retinol stability at 5 °C were stored in the dark. The amount of retinol acetate in the samples and controls was monitored at frequent time intervals over 10 days (Figure 7.1).

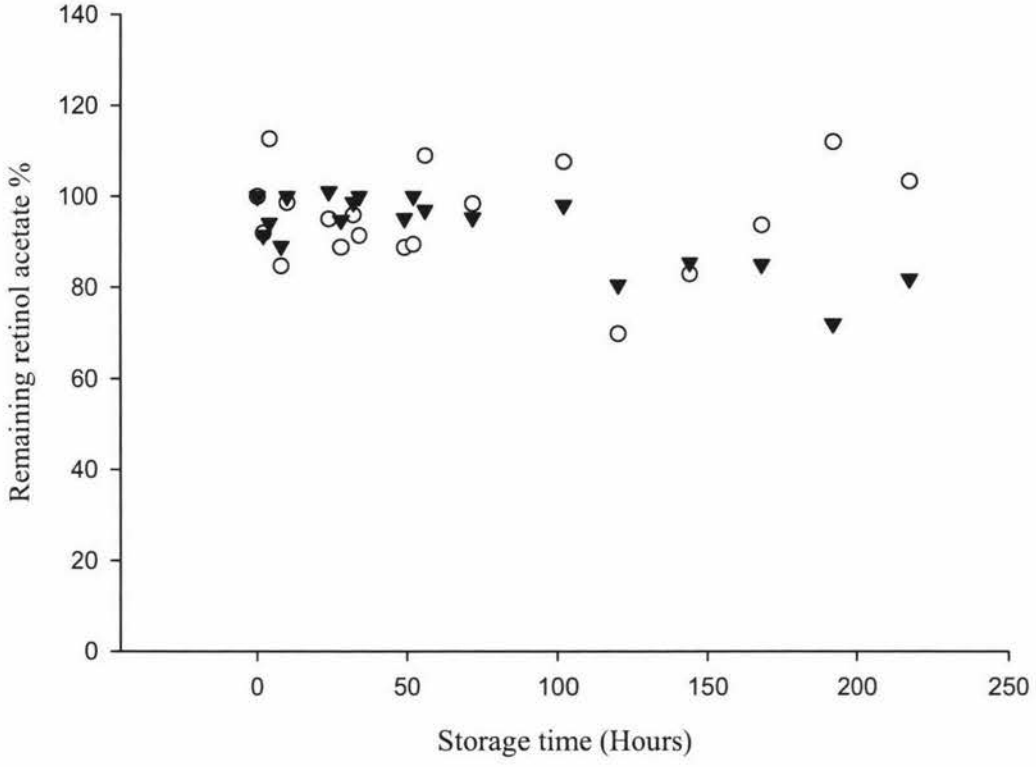

Figure 7.1: Percentages of retinol acetate remaining in the control (○) and sample (▼) stored at 5 °C in the dark, with an initial retinol concentration of 37.5 μg/ml.

The results showed significant variations (± 20%) in the measured retinol concentrations over this period rather than the expected decline in retinol concentration. Similarly, deBoer *et al.* (1983) who used fluorometric analysis, also reported the measured amount of vitamin A in sample was higher than the initial concentration, suggesting that some variations in measured vitamin A are common

regardless of the method. None of the samples or controls indicated a clear decline in retinol acetate concentration, throughout the completed monitoring period (200h). These results suggest that retinol degradation at 5 °C occurs over a period longer than 10 days, and future experiments should be continued for a significantly longer period to reliably identify any degradation trend.

7.2.2 Main study

Based on the preliminary studies, longer time intervals were selected for the main experiment. These intervals were chosen depending on the initial concentration, as higher concentrations were expected to degrade more slowly and therefore required a longer monitoring time. Statistical analysis of the results was performed using a general linear model procedure, developed by SAS -V8 institute inc. (Cary, NC) and the model included the storage time, concentration and the difference between the degradation trends of the control and sample.

Duplicates of the samples containing initial retinol acetate concentrations of 12.5, 25 and 37.5 μg/ml were stored at 5 °C, and the retinol acetate concentration was monitored at frequent time intervals over 6 weeks. The percentages of retinol acetate left in the samples were plotted against time (Figure 7.2, 7.3, 7.4).

7.2.2.1Retinol acetate stored at 5 °C- 12.5 μg/ml

During the first 9 days of the storage, the retinol acetate concentration of the samples showed a decline; approximately 75 % of the initial retinol acetate content was retained. The control displayed variations in the measured retinol content that was similar to the observation made in the preliminary 5 °C trial, presented in section 7.2.1.

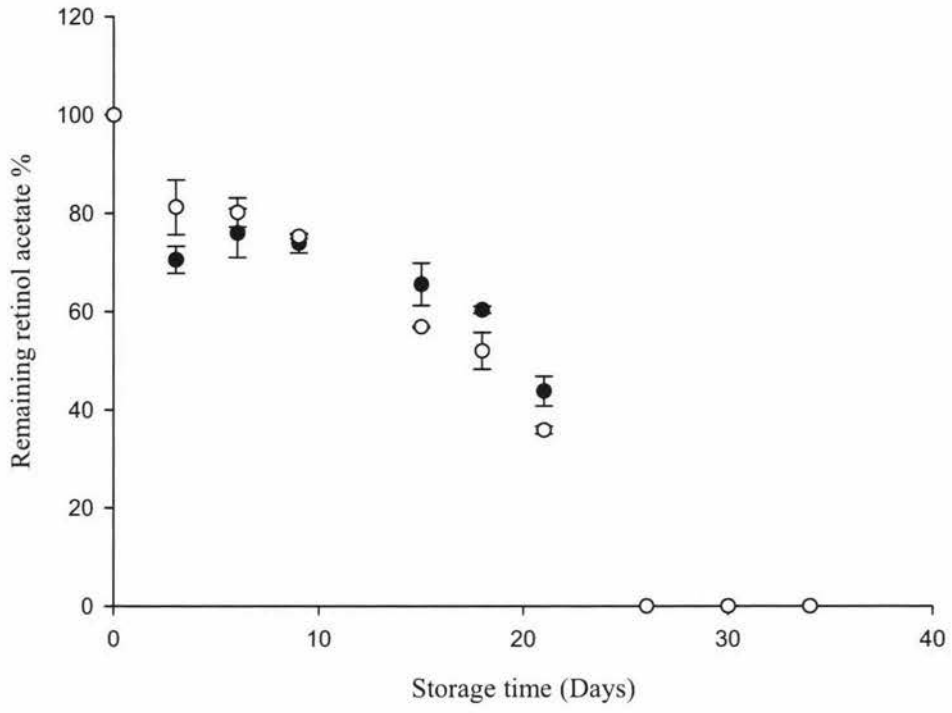

Figure 7.2: Percentages of retinol acetate remaining in the control (●) and sample (○) with initial concentration of 12.5 μg/ml, stored at 5 °C for a period of 34 days.

The control showed a slow decline in retinol acetate concentration by about 15 days of storage, but between 18 and 21 days a noticeable reduction in the concentrations occurred in both the control and sample. The control retained approximately 60 % of the initial retinol acetate, as compared to ~40 % retained by the sample during the first 21 days of storage at 5 °C. Interestingly, after 26 days of storage, total degradation of retinol acetate had occurred in both the sample and control. The longer retention of the retinol acetate in the control shown in this trial indicated the improved stability of vitamin A, in absence of light and higher storage temperature (compared to the results in section 5.2). This is particularly important considering the low initial concentration. On the other hand, the total loss of retinol acetate in both the control and sample at the same time (by 26 days) suggested that the presence of the WPI has a minimal effect on the retinol acetate stability at this concentration.

Statistical analysis revealed the degradation slopes to be significant ($p < 0.0001$) indicating the loss of retinol during storage was statistically significant. However, no

significant difference between the vitamin A loss in the control and sample was found. Thus the effect of WPI on the stability of retinol acetate was not statistically significant at this concentration ($p > 0.05$).

7.2.2.2 Retinol acetate stored at 5 °C- 25 μg/ml

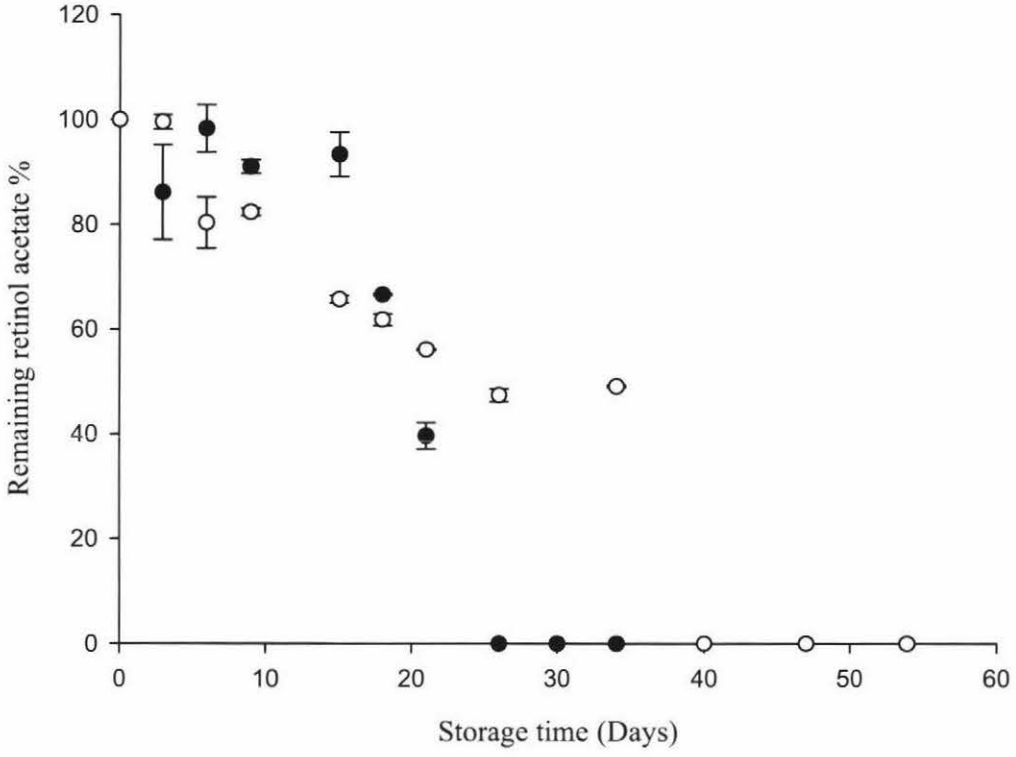

Figure 7.3: Percentages of the retinol acetate remaining in the control (•) and sample (○) with initial concentration of 25 μg/ml, stored at 5 °C over 40 days.

As observed in the earlier experiments, there appeared to be a degree of fluctuation in measured retinol acetate for both the control and sample during the first 10 days of the storage. By 15 days of storage, the control continued to retain 80-95 % of the initial retinol acetate concentration while the sample had lost ~ 30 %. However, a considerable loss of retinol acetate started to appear in the control by day 18, and by day 21 only ~ 40 % of the initial retinol content was remaining in either the sample or the control. This loss continued in the control, and by day 26 no retinol acetate was left. This zero reading was confirmed by subsequent analysis on the days 30 and 34. In contrast, the sample (which contained WPI) showed a gradual decline in retinol

acetate concentration up to day 26, followed by an apparent plateau at ~50 % between day 26 and 34. However, a complete loss of retinol acetate was observed by day 40.

The differences between the degradation trends for the control and the sample were statistically significant. The initial retinol acetate concentration and time had a p value of < 0.0001, indicating a statistically significant amount of retinol degradation had occurred in both the control and sample. More importantly, the difference between these degradation trends was also statistically significant ($p< 0.0003$). In other words, overall the sample retained a significantly more retinol acetate than the control. This suggests the presence of the WPI has a statistically significant protective effect on retinol acetate stored at 5 °C for an initial retinol acetate concentration of 25 μg/ml.

7.2.2.3 Retinol acetate stored at 5 °C- 37.5 μg/ml

The samples containing retinol acetate concentration of 37.5 μg/ml demonstrated much higher stability than the lower concentrations, with total loss of retinol acetate taking 54 days. The sample retained almost 80 % of the initial retinol acetate during the first 9 days of storage. This is inline with the results obtained during the preliminary study presented at the beginning of this chapter. After a month of storage, the sample still retained > 50 % of the initial retinol acetate, a significant improvement on the experiments using lower retinol acetate concentrations. The amount of retinol acetate seemed to plateau at ~ 50 %, between days 26 and 34, but a gradual degradation was observed during the next 12 days until 25 % of the retinol acetate was left, after 48 days of storage at 5 °C. Total loss of retinol acetate in the sample was found after 54 days. It is important to note that the total loss may have occurred anytime between days 48 and 54.

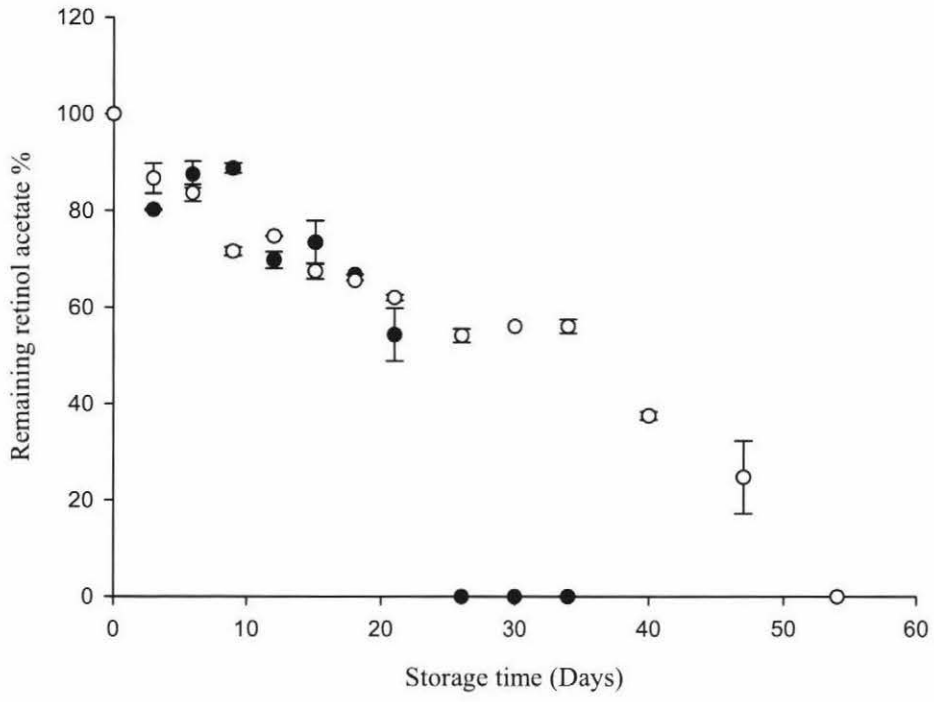

Figure 7.4 Percentages of retinol acetate left in the control (•) and sample (○) with initial concentration of 37.5 μg/ml, stored at 5 °C over 54 days.

The measured retinol acetate in the control fluctuated between 80 –90 % over the first 10 days of storage at 5 °C. This period was followed by a decline in retinol acetate content with ~ 50 % remaining after 21 days. Total loss of retinol acetate had occurred by day 26. Further analysis was carried out on the days 30 and 34, which confirmed no retinol was left in the controls.

Statistical analysis of the degradation trends found that the initial retinol acetate concentration and the degradation time were statistically significant ($p < 0.0001$). The difference between the two degradation trends was also significant ($p< 0.01$), further supporting the hypothesis of WPI having a protective effect on the retinol acetate during the storage at 5 °C, at this concentration.

7.2.3 Discussion

Retinol acetate in both the control and sample remained stable during the first 9 days of storage at 5 °C, regardless of the initial concentration. A certain degree of stability was expected due to the low temperature and the absence of short-wavelength light (Woolard *et al.*, 2003). However, the retinol acetate content started to decline in both the sample and control after 10 days of storage.

Regardless of the initial retinol concentration, the controls demonstrated similar degradation trends. The typical progression was a fluctuation in retinol acetate concentration around 80-90 % of the initial concentration over the first 10 days, followed by a rapid loss. The controls retained ~40-50 % of the initial retinol acetate approximately up to 21 days of storage at 5 °C, despite the dramatic losses observed at the beginning and the end of the experiment. Similar results were reported in milk stored at 5 °C (Woolard *et al.*, 1983), where rapid loss of vitamin A occurred at the beginning and the end of the degradation trend.

The sudden total loss of retinol acetate in all the controls that occurred by day 26 suggested that the initial retinol concentration had no effect on its stability in aqueous environments. There were no apparent external reasons for this dramatic degradation. It is possible that some factor present in the control was stabilising the retinol acetate in the aqueous phase for up to 21 days, but after this time lost its effectiveness. The most likely candidate for such a factor such is the antioxidant. Any antioxidant that is frequently exposed to the oxygen is likely to lose its effectiveness (Llyod *et al.*, 2004). During the 26 days of storage, all the vials were opened at least 7 times to obtain the aliquots to analyse, allowing the headspace to be replenished with oxygen. As the aliquots were removed the remaining volume of the control decreased, while the headspace air volume increased. Reducing the headspace oxygen has been shown to considerably improve stability of fat-soluble vitamins such as vitamin A (deBore *et al.*, 1984; Llyod *et al.*, 2004; literature review section 2.2.1.1). Once the added antioxidant had been oxidised from the control sample, there was nothing to protect the retinol acetate from autooxidation, and it would degrade rapidly.

An alternative explanation is that the relatively constant retinol acetate concentration observed during the first 10 days may indicate a "lag phase" representing the time required to build up sufficient reactive intermediates for autoxidation of vitamin A (Lau *et al.*, 1986). Once a certain concentration of these intermediates (radicals) was reached, it could trigger a rapid degradation of retinol via a radical mechanism (refer to literature review section 2.2.1).

It is possible that a combination of these factors may have been responsible for the sudden retinol acetate loss that was observed in the controls. This phenomenon is important in terms of a vitamin A fortified beverage, particularly if it is not completely consumed on one occasion. Re-opening a product could subject the vitamin A to frequent exposure to oxygen, replenishing the oxygen supply in the headspace and thus using up antioxidants present, as well as potentially shortening any "lag phase" through providing more oxygen for the preparation of reactive intermediates. This implies any vitamin A in a fortified product may undergo more rapid degradation after opening, and thus should be consumed over a relatively short period. The presence of WPI may act to protect the retinol acetate either by providing an additional antioxidant effect, or by preventing the retinol acetate oxidation through direct binding to the proteins.

Overall, the samples, which contained 1 % WPI appeared to be more capable of stabilising retinol than the controls without any WPI. Figure 7.5 compares the degradation trends of the samples with different initial retinol acetate concentrations. Samples with initial concentrations of 25 and 37.5 μg/ml were able to retain retinol acetate during storage at 5 °C for a longer period than the controls. WPI was the only extra factor that was present in the sample compared to the control, implying that WPI may play a role in the stabilisation of retinol acetate during storage at this temperature.

It is known that vitamin A can bind to certain WPI (Schofield, 1999). Retinol binds to β-Lg at a hydrophobic internal binding site (Kontopidis *et al.*, 2004) as well as at a number of external binding sites (Monaco *et al.*, 1997). BSA also binds to vitamin A, enhancing the solubility of the vitamin in an aqueous environment (Futterman *et al.*,

1972). The observed differences between samples and controls suggest that when the retinol acetate was added to the samples, it may have bound to the whey proteins, which subsequently helped stabilise the vitamin in the aqueous phase.

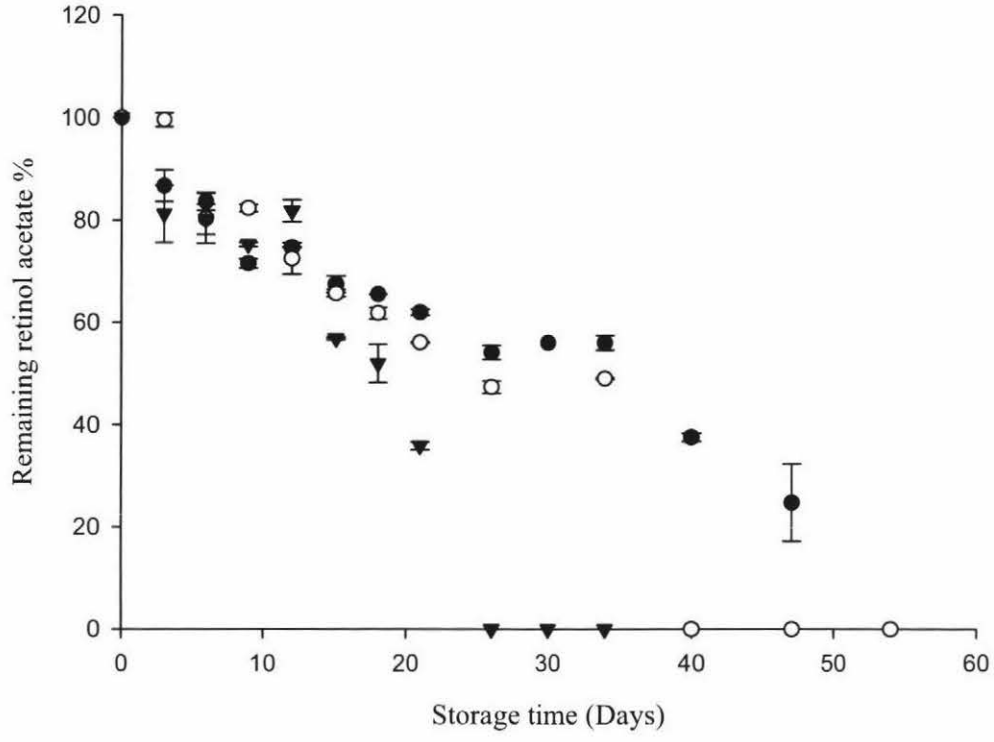

Figure 7.5: The degradation of retinol acetate in samples (containing WPI) with initial concentrations of 12.5 (▼), 25 (○) and 37.5 (●) μg/ml during the storage at 5 °C.

Higher retinol acetate concentrations were stable for a much longer time than the samples with lower concentration. The time for complete loss of the retinol acetate increased from 26 to 54 days, when the concentration was increased from 12.5 to 37.5 μg/ml. This concentration effect may be attributed to a possible limitation of autoxidation, which may also explain the greater losses of retinol acetate observed at the lowest initial concentration where WPI did not seem to improve the retinol stability significantly.

In conclusion, aqueous solutions with initial retinol acetate concentrations of 25 and 37.5 μg/ml were significantly more stable when WPI was included in the system.

7.3 Storage at 40 ^{0}C

The effect of WPI on vitamin A degradation during storage at 40 °C was studied using samples and controls containing initial retinol acetate concentrations of 12.5, 25 and 37.5 μg/ml. All samples and controls were stored in the dark at 40 °C with pyrogallic acid added as the antioxidant. Retinol acetate concentrations were monitored over a period of 0 to 72 hours, using frequent time intervals. These time intervals were based on the initial retinol acetate concentration, as lower concentrations were expected to degrade faster. The degradation trends of controls and samples were analysed using SAS-V8 institute inc. (Cary, NC), using a general linear procedure with a log transformation. The statistical model included the storage time, the retinol concentration and the difference between the degradation observed in the sample and control.

7.3.1 Storage of retinol acetate at 40 °C- 12.5 μg/ml

Rapid losses occurred in both the control and sample containing 12.5 μg/ml of retinol acetate during the storage at 40 °C (Figure 7.6). During the first 3 hours of storage, approximately 25 % of retinol acetate was lost regardless of the presence of WPI. These losses were increased to 50- 60 % within 5 hours of storage, although the sample appeared to retain slightly more retinol acetate than the controls. The degradation of retinol acetate in the sample was slow between 5 and 12 hours of storage, while the control continued to degrade rapidly. Nevertheless, the total loss of retinol acetate occurred in both the control and sample after 14 hours of storage at 40 °C, indicating the strong effect of this storage temperature on the stability of retinol acetate.

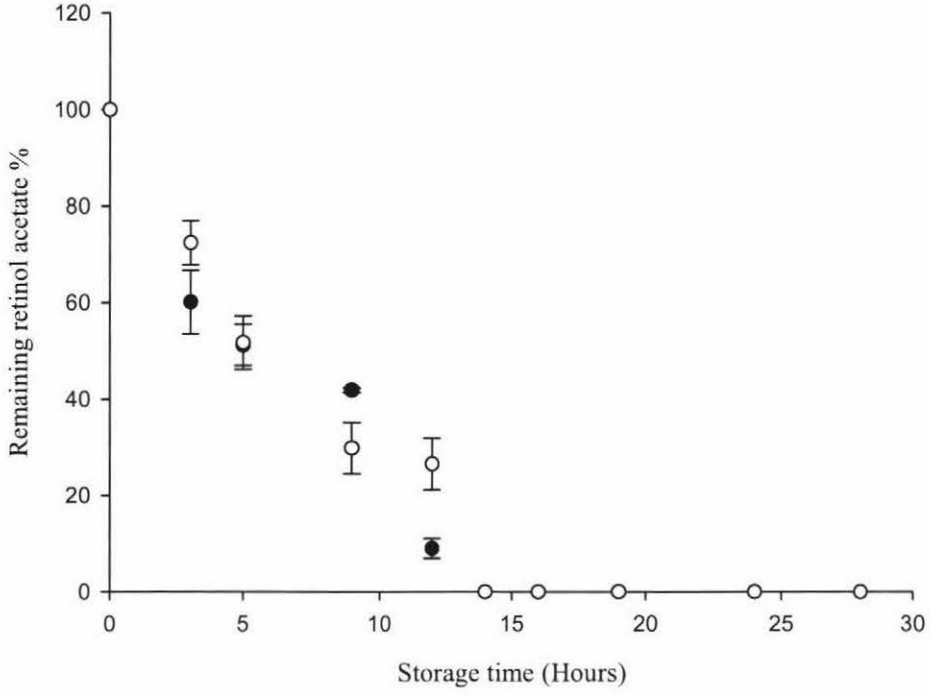

Figure 7.6: Percentages of retinol acetate remaining in samples (○) and controls (●) with an initial concentration of 12.5 μg/ml, stored at 40 °C.

Statistical analysis of the two degradation trends showed no statistical significant difference ($p > 0.05$) between the control and the sample. This suggested that the presence of WPI did not have a statistically significant effect on retinol acetate degradation, at this concentration. However, the initial retinol acetate concentration and the time were both significant ($p < 0.0001$).

7.3.2 Storage of retinol acetate at 40 °C- 25 μg/ml

The degradation of retinol acetate in the sample and the control with an initial concentration of 25 μg/ml occurred more rapidly in the first few hours of storage at 40 °C (Figure 7.7). Almost 50 % of the initial retinol acetate had degraded in both the control and sample after 12 hours. By 16 hours of storage at 40 °C, the control had

lost ~ 60 % of the initial retinol acetate, while the sample had lost ~ 50 % suggesting a slight deviation in the degradation trends.

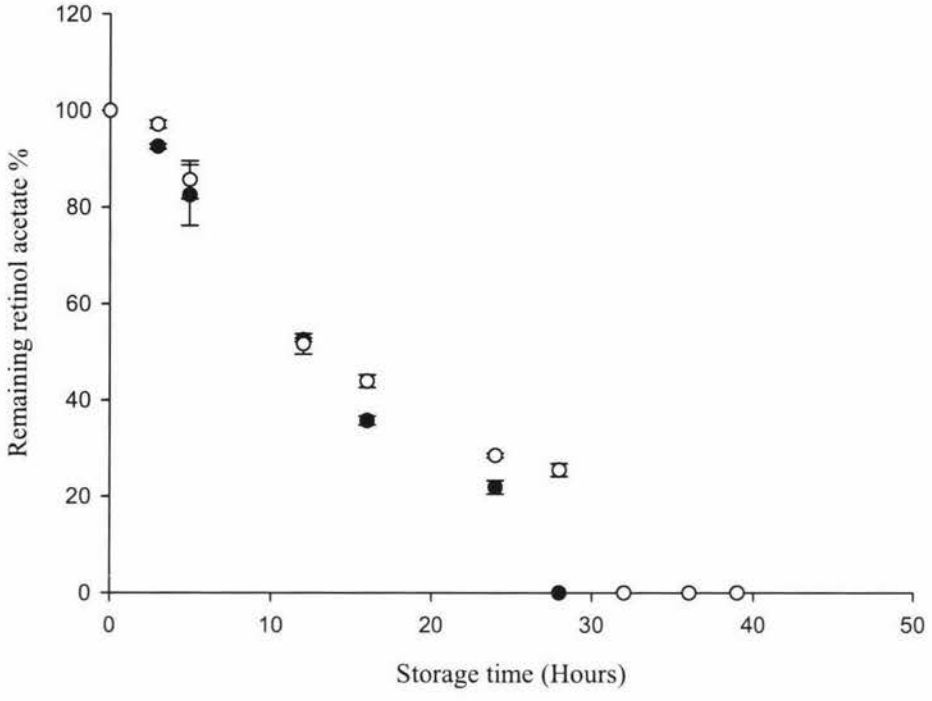

Figure 7.7: Percentages of remaining retinol acetate in samples (○) and controls (●) containing initial concentration of 25 μg/ml stored at 40 °C.

These rates of degradation in the sample and control became more clearly differentiated during the next 10 hours of exposure. The decline in retinol acetate content in the sample slowed, while the control continued to show a rapid loss. After 28 hours of storage, the control (with no WPI) had lost all the retinol acetate, while the sample (with WPI) still retained ~25 % of the initial retinol acetate content. The latter indicated that retinol acetate was more stable in the presence of WPI, during storage at 40 °C.

Statistical analysis showed the degradation time and the concentrations were statistically significant ($p < 0.0001$). However, despite the improved stability of

retinol acetate in the presence of WPI observed in the graph, the difference between the two degradation trends was not significant ($p > 0.05$).

7.3.3 Storage of retinol acetate at 40 °C- 37.5 µg/ml

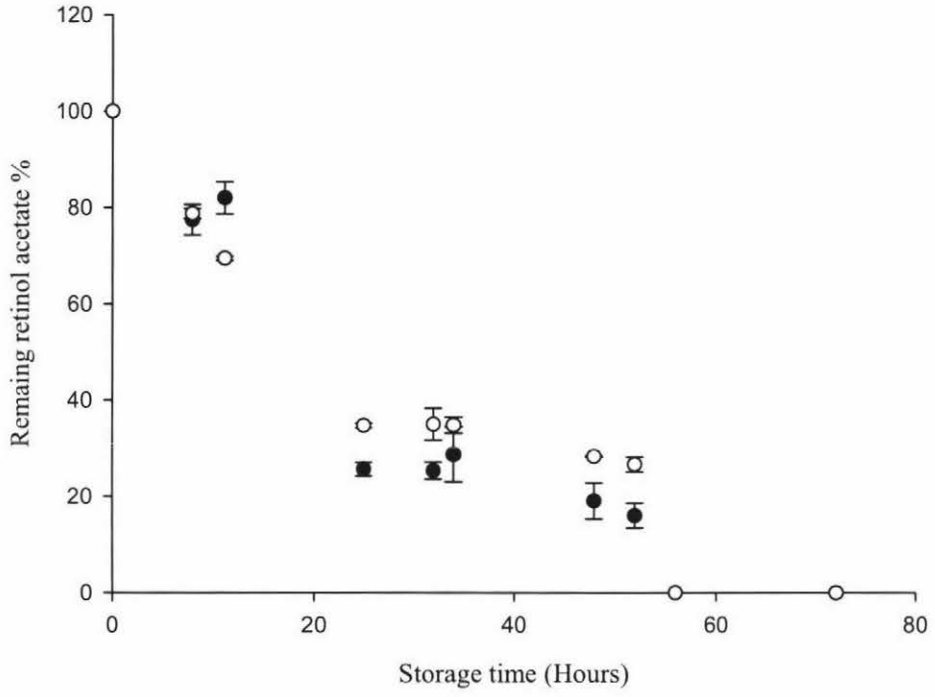

Figure 7.8: Percentages of retinol acetate remaining in samples (○) and controls (●) that contained initial concentration of 37.5 µg/ml, stored at 40 °C.

The instability of retinol acetate at 40 °C was apparent (Figure 7.8) even at the highest initial concentration (37.5 µg/ml). More than 50 % of the initial retinol acetate had been lost in both the control and sample after 25 hours of storage at 40 °C. A slower degradation of retinol acetate was observed within the second 24 hour period, in both the sample and the control. The sample retained slightly more retinol acetate than the control during this time. This indicates that WPI may have improved the retinol acetate stability at 40 °C. However, after 56 hours of storage at 40 °C, 100 % loss of

retinol acetate occurred in both the sample and control, which shows the limited capacity of the WPI in stabilising retinol acetate during storage at high temperatures.

Results of the statistical analysis were similar to those of the earlier concentrations. The initial retinol concentration and the degradation time were significant ($p< 0.0001$). But there was no significant difference ($p> 0.05$) between the trends of the control and the sample.

7.6 Discussion

All the samples and controls with different initial concentrations of retinol acetate showed rapid losses of the retinol acetate during the storage at 40 °C. The stability of retinol acetate was significantly affected by the increased storage temperature when compared to the observations made during storage at 5 °C. A certain degree of stability was expected due to the absence of short-wave length light; however, the higher storage temperature appears to have had a very large effect. Increasing the storage temperature above the ambient temperature (Woolard *et al.*, 1985) in the absence of the light has been shown to increase the loss of vitamin A (deBoer *et al.*, 1984). Vidal-Valverde *et al.* (1992) reported similar observations with a rapid decrease of retinol concentrations in milk samples stored at 40 °C compared to samples stored at 30 °C after 1 month storage. If such rapid losses were observed in milk, containing 3.6 % of fat (known to have a protective effect on vitamin A (Lau *et al.*, 1986)), it seems not surprising that retinol acetate in an aqueous environment containing no fat was extremely unstable and degraded rapidly.

All three retinol acetate concentrations showed almost similar degradation trends during storage at 40 °C. The degradation trends can be explained as a large loss of the vitamin during the earlier stages of storage, followed by a reduced rate of degradation in the latter stage. These results were similar to the observations made by deBoer *et al.* (1984), Woolard *et al.* (1985) and LeMagure *et al.* (1986) who studied the stability of vitamin A at a range of storage temperatures.

Higher retinol acetate concentrations were stable for a much longer time than lower concentrations in both the sample and control. The stability of the controls indicates

that retinol can be stable at higher concentrations regardless of the presence of a carrier. The time for complete loss of the retinol acetate increased from 12 to 52 hours, when the concentration was increased from 12.5 to 37.5 μg/ml. This concentration effect may be attributed to a possible limitation of autoxidation. The degradation trends in the 3 retinol acetate concentrations show that as the concentration increased, the "lag phase" has become longer (Figure 7.9).

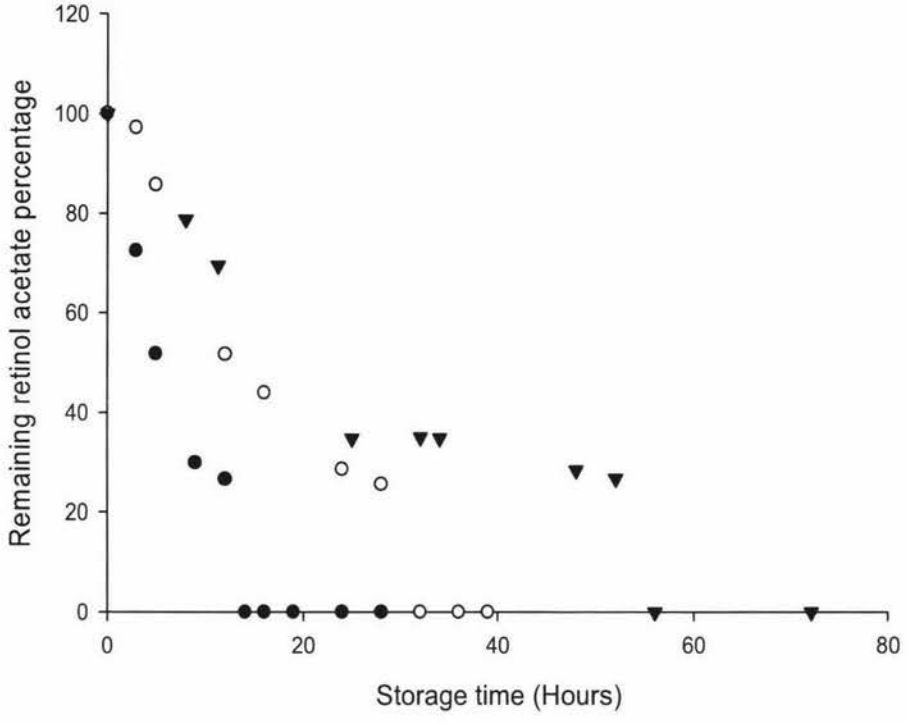

Figure 7.9: The degradation of retinol acetate remaining in samples (with WPI) that contained initial retinol concentrations of 12.5 (●), 25 (○) and 37.5 μg/ml (▼)stored at 40 °C.

The storage at 40 °C is unlikely to affect the thermal stability of WPI, as all major whey proteins have thermal denaturation temperatures above 40 °C (Jelen *et al.*, 1999). Schofield (1999) has reported that the binding capacity of reinol acetate to WPI is not affected at 45 °C. However, any retinol that was not bound would degrade rapidly at this temperature.

Although a partial loss of retinol may have occurred as a result of the elevated storage temperature, it is possible that a combination of factors could have contributed to the speed of the loss. As discussed in the section 7.2.3, in order to obtain the aliquots for analysis each control and sample vial had to be opened a number of times, reintroducing oxygen into the vial headspace. Renewing the oxygen supply may have exhausted the antioxidant capacity (Llyod *et al.*, 2004). As a result, until the oxygen was depleted, the retinyl esters may have been exposed to continuous oxidative stress (Woolard *et al.*, 1985).

In conclusion, although the effect of WPI on retinol acetate storage at 40 °C was not statistically significant, there was a slightly higher retention of retinol acetate in the samples at concentrations > 25 μg/ml, which may be attributed to binding to WPI (Schofield,1999).

CHAPTER 8

OVERALL DISCUSSION AND RECOMMENDATIONS

8.1 Discussion

The approach taken in this study enabled us to develop a reliable method to extract retinol from aqueous based samples and to observe degradation trends of vitamin A in aqueous phased WPI. Saponification was found to be a reliable method to extract retinol from WPI in aqueous systems despite its lengthy procedure.

It is known that vitamin A can bind to certain whey proteins (Schofield *et al.*, 1999). Bovine β-Lg consists of eight anti-parallel β-sheets that form a calyx, which is flanked on one side by an α-helix, to form the hydrophobic pocket (Dufour *et al.*, 1991). Retinol binds to β-Lg at an internal binding site located at the hydrophobic central calyx (Kontopidis *et al.*, 2004), as well as to a number of external binding sites (Monaco *et al.*, 1997). BSA also binds to vitamin A, enhancing the solublity of the vitamin in an aqueous environment (Futterman *et al.*, 1972). The observed differences in retinol degradation in the presence of WPI suggest that when the retinol acetate was added to the samples, it may have bound to the whey proteins, and that this binding subsequently helped stabilise the vitamin in the aqueous phase.

This research clearly showed that retinol acetate is extremely sensitive to light, storage and processing temperatures. The degradation of retinol was exponential when stored at 40 °C and in the presence of light. WPI appeared to have a limited protective effect to retinol acetate during exposure to light and higher storage temperature.

In contrast, WPI showed a significant protective effect on retinol acetate, when stored in the absence of light and when stored at 5 °C. There was also a significant protective effect during pasteurisation (72 °C for 15 seconds). WPI failed to show any noticeable effect on retinol acetate stability during a UHT treatment (144 °C for 4

seconds) with all the vitamin being lost from samples and controls. This lack of protective effect may be attributed to the thermal instability of the whey proteins, which have thermal denaturation temperatures below 87 °C.

The stoichiometry of the binding of retinol to β-Lg is known to be 1:1 (Futturman *et al.*, 1972; Dufour *et al.*, 1994). Since β-Lg comprises approximately 50% of the protein in WPI, the solutions used in these experiments should contain ~ 5 x 10^{-3} μg/ml or ~2.688 x 10^{-7}mols of β-Lg. In theory, this could provide protection for an equivalent number of moles of retinol acetate. The retinol acetate concentrations used in this work were between 3.8051 x 10^{-5} to 1.1413 x 10^{-4} mols (refer to the appendix for calculations), significantly higher than the β-Lg.

After exposure to a pasteurisation treatment, between 4.85 x 10^{-8} and 3.531 x 10^{-8}mol of retinol remained in the samples, more than the amount that could have been bound to the β-Lg. This suggests that the retinol may have bound to other proteins present in the WPI, including BSA.

Higher initial retinol acetate showed more significant protective effect from the WPI in an aqueous environment. Poor stability was shown at the lowest initial concentration (12.5 μg/ml) during exposure to light and after storage at 5 °C and 40 °C. This suggests that at the lower retinol concentrations, the protective effect of the WPI may have been overpowered by the oxidative stress. It is possible a small amount of retinol dispersed in a relatively large volume could have been more susceptible to oxygen. The high reactivity of retinol and its derivatives may be too great to protect it all from residual oxidants, whether exogenous or co-existing within the milk matrix (Woolard *et al.*, 1985).

It is important to note that these results were obtained using retinol acetate that was added to the samples, and then converted to retinol by saponification. This means that the analysis was based on the assumption of 100 % conversion of retinol acetate to retinol. Saponification is the universally accepted method (Ball, 1999) for retinol quantification; however it may be possible that there was a rare occurrence of not achieving the 100 % conversion, which may have given a lower result than the actual amount of retinol acetate present.

8.2 Recommendations

One of the main issues associated with quantifying retinol is the method sensitivity. Due to the poor sensitivity of the method the initial retinol acetate concentration used in this experiment was considerably higher than the current vitamin A fortification of dairy products in New Zealand. Considering the potential use in food fortification, it is recommended to use initial retinol concentrations that comply with the food fortification regulations in the future work that is carried out with respect to stabilisation of vitamin A.

It is important to negate any possible vitamin A oxidation that is likely to result by indirect exposure to oxygen, in future work. It is strongly recommended to nitrogen flush the samples and controls before and after obtaining the aliquots for analysis. This would prevent any possible vitamin A degradation that is likely to occur due to the re-introduction of the oxygen into the vials. It may also be useful to monitor the antioxidant capacity in the samples used for storage stability studies to clarify any doubts of the antioxidant wastage or inefficiency. Antioxidant assays to measure concentration of the antioxidant could be useful in such attempts.

Based on the retinol stability observed in the presence and in the absence of light, it would be interesting to investigate the effect of other factors such as use of a label and light impermeable packaging to improve the retinol stability. Covering a major portion of a product container could effectively minimise light induced oxidation of vitamin A.

During this research the retinol concentration was the variable while the carrier (WPI) was invariable. The results of the pasteurisation suggested a possible effect of the WPI concentration. However further investigation is required to clarify this and therefore it may be worth to study the impact of the carrier concentration in future work. In future work the amount of WPI could be varied (1 to 5 %) to study any possible saturation effect of retinol on WPI while the retinol concentration remain constant. This may also be useful to clarify the limited binding capacity of the WPI that may have affected the results of this research.

It is also strongly recommended to maintain a pH that could optimise retinol binding to WPI to further improve the retinol stability. This is particularly important as the pH has a strong influence on stability of WPI during high temperature processing.

8.3 Conclusion

In conclusion, a small improvement in the stability of retinol acetate in aqueous environment can be achieved using WPI, especially at higher initial retinol concentration and during treatments such as pasteurisation. This is most likely to be of benefit when used in combination with other techniques designed to minimise the exposure to potentially harmful factors (such as light and higher storage temperatures)

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Appendix

Stoichiometry calculations

β-Lg: Retinol stoichiometry is 1: 1

Therefore 1 mol of β-Lg should bind with 1 mol of retinol;
Based on 1% WPI solution;
Concentration of the WPI = 0.010 g/ml
But 50% of WPI is β-Lg;
Therefore concentration of the β-Lg = 0.010 x 50%
= 0.005g/ml

MW of β-Lg ~ = 18.6 kg/mol (Bon *et al.*, 1999)
Therefore the number of moles of β-Lg in 1ml;

$$= \frac{0.005\ g}{18.6 \times 10^{3} g}$$
$$= 0.0002688 \times 10^{-3}$$
$$= \mathbf{2.688 \times 10^{-7}\ mols}$$

Number of retinol acetate moles in the sample with the highest retinol concentration

Highest concentration **37.5 μg/ml**;
Therefore 1 ml should have 37.5 μg of retinol, which is 0.0375mg
$0.0375 \times 10^{-3} g$
$=37.5 \times 10^{-6} g$

MW of retinol acetate = 328.5g

Therefore number of moles of retinol acetate $= \frac{37.5 \times 10^{-6}\ g}{328.5 g}$
$= 0.1141 \times 10^{-6}$
$= 1.141 \times 10^{-7} mol$

Therefore WPI is not likely to be the limiting factor according to the stoichiometry as there is an abundance of β-Lg is present.

At 12.5 μg/ml;
In 1 ml the number of retinol acetate mols;

$$= \frac{12.5 \times 10^{-6}\ g}{328.5 g}$$

$= 0.038051 \times 10^{-6}$

$= 3.8051 \times 10^{-8}$mol

At 25 μg/ml;

In a 1 ml the number of retinol acetate mols;

$$= \frac{25 \times 10^{-6}}{328.5g}$$

$= 0.076103 \times 10^{-6}$

$= 7.6103 \times 10^{-8}$mol

At 37.5 μg/ml; $= 1.1413 \times 10^{-7}$mol

Processing temperature

The constant amount of retinol being protected was ~ 0.245, 0.319, 0.2320 μg/ml of retinol at 12.5, 25, 37.5 μg/ml. Therefore, these values in terms of mols are;

12.5 μg/ml

0.2451μg in a 1ml ;

Considering the dilutions = 0.2451μg x 50 = 12.555 μg/ml

Therefore, the number of mols = $\frac{12.555 \times 10^{-6}}{328.5}$

$= 0.03730 \times 10^{-6}$mols $= 3.73 \times 10^{-8}$mol

25 μg/ml

0.319 μg in a 1ml ;

Considering the dilutions = 0.319 μg x 50 = 15.95 μg/ml

Therefore, the number of mols = $\frac{15.95 \times 10^{-6}}{328.5}$

$= 0.04855 \times 10^{-6}$mols $= 4.85 \times 10^{-8}$mol

37.5 μg/ml

0.2320 μg in a 1ml ;

Considering the dilutions = 0.2320 μg x 50 = 11.6 μg/ml

Therefore, the number of mols = $\frac{11.6 \times 10^{-6}}{328.5}$

$= 0.03531 \times 10^{-6}$mols $= 3.531 \times 10^{-8}$mol

$< 2.688 \times 10^{-7}$ mols Of β-Lg

Therefore, more β-Lg than the amount of retinol left after pasteurisation.

Light trials

Based on the plateau at each concentration;

37.5 µg/ml
0.156769 In a 1ml ;

Considering the dilutions = 0.156769 µg x 50

= 7.83845 µg/ml

Therefore, the number of mols = $\frac{7.83845 \times 10^{-6}}{328.5}$

$= 0.023861 \times 10^{-6}$mols
$= 2.38 \times 10^{-8}$mol

25 µg/ml

0.116468µg in a 1ml ;

Considering the dilutions = 0.116468µg x 50

= 5.8324 µg/ml

Therefore, the number of mols = $\frac{5.8324 \times 10^{-6}}{328.5}$

$= 0.017727 \times 10^{-6}$mols

$= 1.77 \times 10^{-8}$

12.5 µg/ml

0.107826 µg in a 1ml ;

Considering the dilutions = 0.107826 µg x 50

= 5.3913 µg/ml

Therefore, the number of mols = $\frac{5.3913 \times 10^{-6}}{328.5}$

$= 0.01641 \times 10^{-6}$mols
$= 1.641 \times 10^{-8}$mol

At 40 °C

37.5 µg/ml

0.156769 in a 1ml ;

Considering the dilutions = 0.156769 µg x 50

= 7.83845 µg/ml

Therefore, the number of mols = $\frac{7.83845 \times 10^{-6}}{328.5}$

= 0.023861×10^{-6}mols

= 2.38×10^{-8}mol

25 µg/ml

0.116468µg in a 1ml ;

Considering the dilutions = 0.116468µg x 50

= 5.8324 µg/ml

Therefore, the number of mols = $\frac{5.8324 \times 10^{-6}}{328.5}$

= 0.017727×10^{-6}mols

= 1.77×10^{-8}

12.5 µg/ml

0.107826 µg in a 1ml ;

Considering the dilutions = 0.107826 µg x 50

= 5.3913 µg/ml

Therefore, the number of mols = $\frac{5.3913 \times 10^{-6}}{328.5}$

= 0.01641×10^{-6}mols

= 1.641×10^{-8}mol