Cholesterol Oxidation Products In Whole Milk Powder:

Analytical, nutritional, processing and toxicological studies.

PhD thesis

by

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Declaration

This thesis is submitted in fulfilment of the requirements for Doctor of Philosophy, by research and thesis. Except where otherwise acknowledged, this work was carried out by the author alone, on a full time basis between October 1992 and January 1997 at the School of Biological Sciences, Dublin City University and The National Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork

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January 24, 1997.

This thesis is dedicated to my Dad whose hypercholesterolemia has fostered in me a longstanding fascination with cholesterol. This is for you Dad.

'Two Roads diverged in a yellow wood,

I took the one less travelled by and

that has made all the difference.....'

Robert Frost (1878 - 1903)

TITLE: Cholesterol oxidation products in whole milk powders:

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ABSTRACT

The possibility that consumption of aged whole milk powders containing significant levels of cholesterol oxides may be detrimental to health has stimulated extensive research. In this study, the effects of feeding regimen and processing technology on the oxidative stability of lipids and cholesterol in whole milk powders were evaluated. Animal feeding regimen had a significant effect ($p < 0.05$) on the levels of total protein, true protein, casein protein and whey protein in standardised milk over the experimental period. Levels of peroxides, thiobarbituric acid reactive substances and cholesterol oxidation products were used as indices of lipid oxidation occurring in whole milk powders. Lipid and cholesterol oxidation increased significantly ($p < 0.01$) after 12 months storage, the predominant oxysterols being 7-ketocholesterol, cholesterol-α-and β-epoxides, 25-hydroxycholesterol and cholestanetriol. A positive correlation was found between the levels of thiobarbituric acid reactive substances and cholesterol oxidation products. Significant decreases in lipid oxidation were observed as a result of high pre-heat treatment of milk prior to processing ($p < 0.001$), vacuum packing of powders in laminated foil bags prior to storage ($p < 0.05$), and storing powders at reduced temperatures (15° C) ($p < 0.05$). Levels of free sulphydryl groups, which possess antioxidant activity in whole milk powders, increased significantly ($p < 0.05$) as a result of a grass plus concentrate-fed diet and the application of high pre-heat treatment of milk. The toxicity of these oxysterols was evaluted using primary cultures of porcine ovarian granulosa cells. The activities of catalase and superoxide dismutase, two antioxidant defense enzymes, were increased significantly ($p < 0.01$) following 24 hour exposure to 2.5 μM concentrations of both cholestanetriol and 25-hydroxycholesterol. Levels of thiobarbituric acid reactive substances remained unchanged under these conditions. Administration of α-tocopherol to the culture medium significantly ($p < 0.01$) improved cell viability and restored catalase and superoxide dismutase activities to control levels. These studies suggest that oxysterols, at levels found in whole milk powders were non cytotoxic.

Abbreviations

ADMI : American dry milk institute
BHA: butylated hydroxyl anisol
BHT: butylated hydroxyl toluene
BSTFA: N,O-bis (trimethylsilyl) trifluoracetamide
CAT: catalase
COPs: cholesterol oxidation products
FAME: fatty acid methyl esters
FFA : free fatty acids
GC : gas chromatography
GPx :glutathione peroxidase
H: hours
HDL: high density lipoprotein
High-heat: high pre-heat treatment
HMG-CoA reductase: 3-hydroxy-3-methylglutaryl CoA reductase
HPLC: high performance liquid chromatography
IDF : International Dairy Federation
LDL: low density lipoprotein
Low-heat: low pre-heat treatment
Min: minutes
MONICA: monitoring risk factors and determinants in coronary heart disease
MS: mass spectrometry
MTT: 3-[4,5-dimethylthazol-2-y]-2,5-diphenyltetrazolium bromide
NADP: nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH :nicotinamide adenine dinucleotide phosphate (reduced form)
NCS: newborn calf serum
NMR: nuclear magnetic resonance
PG: propyl gallate
PV: peroxide value
sec: seconds
SOD: superoxide dismutase
SPE: solid phase extraction
TBARS: thiobarbituric acid reactive substances
TBHQ: tert-butylhydroquinone
USP: United States Pharmaceutical
VLDL: very low density lipoprotein
WPNI: whey protein nitrogen index

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Go raibh mile mile maith agaibh go leir!!!!

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CHAPTER 1

LITERATURE REVIEW

The production of whole milk powder in Ireland amounts to approximately 36,000 metric tonnes per annum (Irish Dairy Board Annual Report, 1995), making a significant contribution to Ireland's £100 billion food industry. The importance of milk and it's various dairy products in the diet of people in Western countries is indicated by the fact that the average consumption of milk (in all it's forms) per person in the EC is approximately 1 litre per day (Mepham, 1987).

Lipid oxidation products are ubiquitous in foods, although much variation exists in the levels present. Although these levels are generally low, the problem of lipid oxidation severely compromises the quality of stored foods and limits their shelf life. Lipid oxidation represents a key barrier in the development of new food products, especially convenience items and processes required in their manufacture. Deleterious changes in foods caused by lipid oxidation include loss of flavour, development of off-flavours, loss of colour, reduction of nutrient value and functionality and the accumulation of compounds which may be detrimental to health. All foods that contain lipids are susceptible to oxidative deterioration, but especially affected are dehydrated foods and those subjected to high temperatures prior to storage, e.g spray-dried whole milk powders, eggs, cheeses and foods fried in frying oils. Specific examples of compounds which are of concern to health include lipid peroxides and the free radicals involved in their formation/propagation as well as several cholesterol oxidation products (COPs) (Addis and Warner, 1992). Given the powerful array of antioxidants available and the recent advances in processing technology, identification of the steps that will reduce or control levels of oxidation products in foods is a major goal for the food industry and regulatory bodies.

The aim of this chapter is to define the composition of milk, to review the manufacture and properties of whole milk powder and to present a state of the art review of the development, control and biological effects of lipid and cholesterol oxidation.

1.1 DEFINITION OF MILK

Milk is defined in the Milk Ordinance and Code recommended by the United States Public Health Service as 'the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which contains not less than 8.25 % of milk-solids-not-fat and not less than 3.25 % of milkfat' (Webb and Johnson, 1974). The distinctive feature of milk as a food lies in the balanced content of essential nutrients which it contains. This has been appreciated for thousands of years and led to the early domestication of docile, herbivorous ruminants, including cows, goats and camels (Mepham, 1987).

1.2 GROSS COMPOSITION OF MILK

The constituents of cows' milk are derived from two sources. Several major components including fat, lactose and proteins and numerous minor components, including a variety of enzymes and sugar derivatives are synthesised within the mammary gland from materials present in the blood plasma, whereas other components such as water, minerals and vitamins are transferred directly from the blood plasma to milk (Kurtz, 1974). The composition of milk determines it's nutritive quality, it's value as a raw material for making food products and it's physical, chemical and functional properties. The principal constituents of milk are shown in Table 1.1.

Table 1.1 Approximate Composition of Bovine Milk.

Component	Average Content Percentage (w/w)
Water	87.3
Fat	3.9
Protein	3.2
Lactose	4.6
Ash	0.7

(Walstra and Jenness, 1984)

1.2.1 Milkfat

Milk lipids provide a major source of energy and essential structural components for the cell membranes of the newborn in all mammalian species (Christie, 1994). They confer distinctive properties on dairy foods that affect processing. In consequence, the composition, structure and chemistry of milk lipids have probably been studied more intensively than those from any other natural source. Bovine milk lipids have received most attention because of their commercial importance. The content and composition of lipids from milks of different species vary with such factors as diet, stage of lactation, number of lactations, breed and season (Hawke and Taylor, 1994).

1.2.1.1 Composition of Milkfat

In the milks of all species studied to date, triglycerides are by far the major lipid class, accounting for 97-98 % of total lipids (Table 1.2). The triglycerides are accompanied by small amounts of di- and monoglycerides, free cholesterol and cholesteryl esters (commonly in the ratio 10:1), free fatty acids and phospholipids. In addition to these, a number of minor simple lipids and glycolipids have been found (Kurtz, 1974; Christie, 1994). Triglycerides consist of a molecule of glycerol to which is esterified three fatty acids, thus the major components of triglycerides are fatty acids, which in milkfat account for over 85 % of the total weight. Monoglycerides and diglycerides contain one and two fatty acids, respectively. Triglycerides in milk are in themselves exceedingly complex, with different types of fatty acid esterified to glycerol and the position at which fatty acids are attached also varying (Fox, 1991). Phospholipids account for about 1 % of total lipid in milkfat. The phospholipids of milkfat consist of several classes (Sonntag, 1979), the major ones being phosphatidylcholine or lecithin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin and plasmalogens. The phospholipids of milk and dairy products are concentrated either in the milkfat globule membrane (MFGM) itself or in other membranous material that is probably derived from the MFGM (Christie, 1994). During processing of milk, the MFGM may be disrupted and the phospholipids are then found in association with the water phase (Mulder and Walstra, 1974). Although they are only a small proportion of the

total lipids in milk, the phospholipids act as detergents and are capable of covering a large surface area, so their content may be of considerable relevance during milk processing. Cholesterol is the principal sterol found in milkfat although lanosterol and small quantities of dihydrolanosterol and β-sitosterol (phytosterols) have also been found (Sonntag, 1979). Approximately 10-15 % of the total cholesterol in milk is believed to be esterified to fatty acids. Linoleic acid has been reported to account for 27 % of total fatty acids in milkfat cholesteryl esters (Patton and McCarthy, 1963). Fresh milk contains free fatty acids (FFA) which increase during storage of raw milk as a result of hydrolysis of the ester linkage by lipase enzymes secreted by psychrotrophic bacteria. Factors affecting FFA levels in milk include animal nutrition, stage of lactation, storage temperature, time of milking and extent of foaming or agitation occurring during milking and milk assembly (Rajeh and Burgess, 1991).

Table 1.2 Composition of individual simple lipids and total phospholipids in milks from various species.

Lipid Class	**Cow**	**Buffalo**	**Human**	**Pig**
	(wt %	of total	lipid)	
Triglycerides	97.50	98.60	98.20	96.80
Diglycerides	0.36	-	0.70	0.70
Monoglycerides	0.03	-	trace	0.10
Cholesteryl esters	trace	0.10	trace	0.06
Cholesterol	0.31	0.30	0.25	0.60
Free fatty acids	0.03	0.50	0.40	0.20
Phospholipids	0.60	0.50	0.26	1.60

(Christie, 1994)

1.2.1.2 Factors affecting milkfat levels and composition

Bovine milk typically contains approximately 3.5 % fat but the level varies widely depending on several factors including breed, stage of lactation, season, nutritional status, type of feed, health and age of the animal and interval between milking (Fox, 1991).

A wide diversity of cow breeds exist contributing to the commercial milk pool, from Holstein and Friesian to the Kerry cow. This has led to a wide variety in milk yield and to a somewhat lesser extent, composition (Walstra and Jenness, 1984). Milkfat composition is influenced strongly by stage of lactation (Palmquist *et al.*, 1993). The proportion of short chain fatty acids (*de novo* synthesis) are low initially, and increase gradually until at least week 8 to 10 into lactation (Palmquist *et al.*, 1993). For any particular population, the milkfat content is highest in winter and lowest in summer. In Ireland, lactational, seasonal, and possibly nutritional effects coincide and the seasonal changes in fat content (and of protein and lactose) are much greater than in other European countries (Fox, 1991). The quality and quantity of animal feed also affects milkfat yield and composition with milkfat composition being readily modified by the feeding regimen (Sutton, 1989; Grummer, 1991; Murphy *et al.*, 1995). The most significant changes in milkfat quality relate to rheological properties, which influence numerous aspects of character and quality of manufactured dairy products (Palmquist *et al.*, 1993). The composition of milkfat depends on the route from which it is derived (Early, 1992). The fat content of milk is also affected by interval of milking, with milkfat increasing continuously throughout the milking process. If a cow is incompletely milked, the fat content of the milk will be reduced and the entrapped fat will be expressed in the subsequent milking, giving an artificially high value for fat content (Fox, 1991).

1.2.1.3 Milkfat globule membrane (MFGM)

Milkfat is secreted as individual globules, each encapsulated within a membrane, the presence of which prevents coalescence of the fat (Fig. 1.1). The fat globule is excreted from the secretary cell in the mammary gland by being 'pushed' through

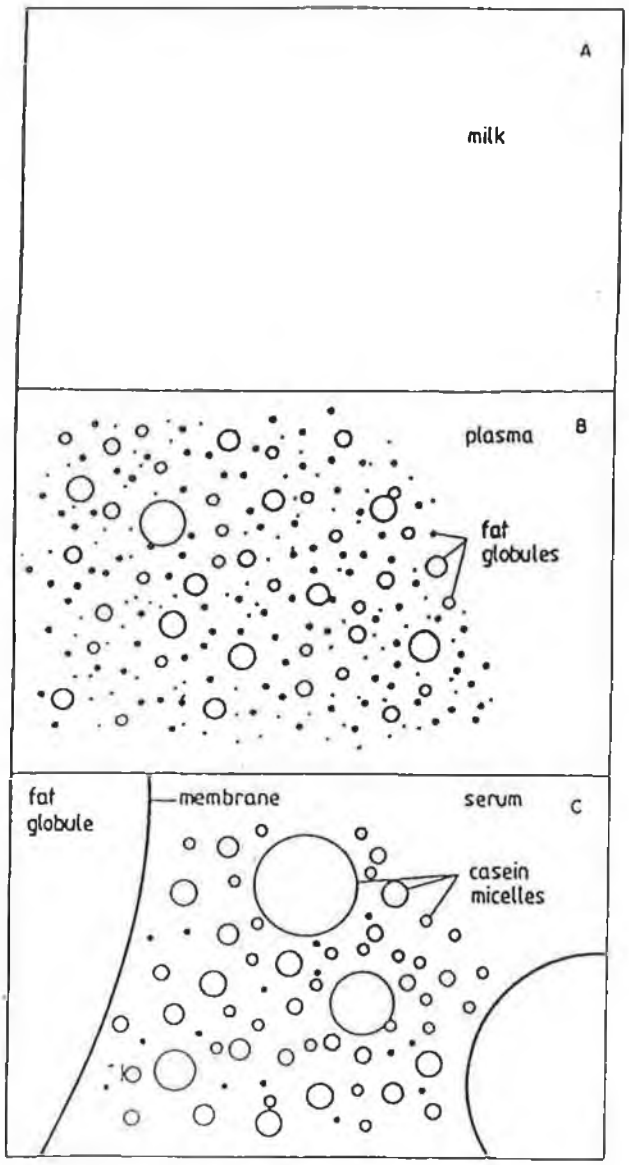


Fig. 1.1 Photomicrograph of milk.

(A) Magnified x 5. Uniform liquid.
(B) Magnified x 500. Spherical droplets consisting of fat. These globules float in a liquid plasma. This is still turbid.
(C) Magnified x 50,000. The plasma contains proteinaceous particles (casein micelles).

the apical cell membrane and becoming surrounded with the membrane in the process. This excretion process has been confirmed by many investigators, although the complete mechanism has still not been fully explained (Brunner, 1974; Keenan *et al.*, 1983). The MFGM serves as a barrier separating aqueous compartments with different solute composition but also serves as the structural base to which certain enzymes and transport systems are bound. Although there is considerable variation (with cell type, species and nutritional status), the typical composition of membranes is approximately 40 % lipid and approximately 60 % protein. The lipids are mostly apolar, composed principally of phospholipids and cholesterol in varying proportions. The phospholipid component of the membrane is particularly rich in polyunsaturated fatty acids, which are liable to undergo oxidation thereby giving rise to 'off-flavours' (Keenan *et al.*, 1983). The lipid molecules appear to be arranged in a bi-layer structure with a total thickness of 7-9 nm (Fox, 1991). The membrane is very sensitive to damage by abrasion. It's whole or partial removal causes the fat globules to coalesce and renders them much more sensitive to lipoprotein lipase, the natural lipase present in raw milk. Lipoprotein action leads to the hydrolysis of milkfat and the liberation of FFA, resulting in the development of an unacceptable rancid flavour and odour. For these reasons, milk must be carefully handled during all stages of transit and storage. To retain the membrane on the globule, the most important single point is to avoid abrasive processes in handling milk. Positive pumping is therefore to be recommended and areas of turbulence are to be avoided (Rajeh and Burgess, 1991).

1.2.1.4 Milkfat Synthesis

Triglycerides comprise approximately 97-98 % of milkfat and fatty acids are the primary constituent. Fatty acids are derived from two sources: (i) *de novo* synthesis in the mammary gland, and (ii) uptake from the circulating blood (Fig. 1.2). The composition of the fatty acids from these two sources is usually very different. e.g. endogenously produced fatty acids are of carbon length C_4 to C_{16}, while a proportion of C_{16}, and virtually all the C_{18} fatty acids arise from blood (Hawke and Taylor, 1994). Estimates of the contribution of blood lipids to milk lipids in the cow vary

from about 35 % to as high as 85 % (Bishop *et al.*, 1969; Annison *et al.*, 1967). Under normal dietary conditions the triglyceride of very low density lipoproteins (VLDLs) and low density lipoproteins (LDLs) appears to be important in providing fatty acids for bovine milkfat synthesis (Glascock and Welch, 1974). The uptake of VLDL triglycerides by mammary tissue involves hydrolysis of the triglycerides by lipoprotein lipase. The lipase appears to be bound to the luminal surface of the endothelium of the blood capillaries and hydrolysis of triglycerides is regarded as a pre-requisite to the uptake of lipid by mammary epithelial cells. Bishop *et al.* (1969) estimated that approximately 30 % of palmitate (C16:0) is synthesised *de novo* by the mammary gland. There is extensive desaturation of stearic acid (C18:0) to oleate (C18:1) on entry into the mammary gland by a desaturase located in the microsomes (Annison *et al.*, 1967). Thus, despite the high 18:0 to 18:1 ratios in triglycerides circulating in the blood of ruminants arising from extensive biohydrogenation of dietary fat in the rumen, desaturation of 18:0 in mammary tissue results in the production of milkfat not markedly dissimilar in 18:1 content to the milkfat of non-ruminants. In general, the nature of the fatty acids taken up from the blood and utilized for milkfat synthesis reflects the composition of the circulating triglycerides. Biohydrogenation and other microbial reactions concerned with lipid metabolism in the rumen ensure a measure of consistency in the nature and level of the fatty acids absorbed in the lower gut, irrespective of the dietary lipid (Hawke and Taylor, 1994).

The significance of feed in influencing fatty acid composition of milkfat is evident in the altered physical and chemical characteristics of milkfat that accompany dietary change (Cullinane *et al.*, 1984; Grummer, 1991). Recent feeding studies (Murphy *et al.*, 1990; 1995) have shown that it is possible to alter the fatty acid composition of milkfat by including ground fullfat soyabeans or ground fullfat rapeseed in the concentrate mixture fed to lactating cows at pasture. The unsaturated C18 fatty acids in these oilseeds are hydrogenated in the rumen to C18:0, which is then absorbed from the intestine and is converted to C18:1 in the mammary gland by a stearic acid desaturase. Murphy *et al.* (1990; 1995) found that increasing the

supply of stearic acid to the mammary gland resulted in an increase in C18:1 in the milkfat, resulting in a softer fat.

Essential to fatty acid biosynthesis in the mammary gland is the provision of a carbon source in the form of acetyl-CoA and the availability of large quantities of reducing equivalents, such as NADPH. Folley and French (1950) showed that ruminant mammary tissue utilised acetate and not glucose for fatty acid synthesis, whereas glucose represented the major carbon source for fatty acid synthesis *de novo* in non-ruminant mammary tissue. Large amounts of acetate, propionate and butyrate, produced by the fermentation of dietary carbohydrates in the rumen are absorbed through the ruminal wall. The utilisation of blood acetate, rather than blood glucose by ruminants as a carbon source in lipogenesis, represented a unique metabolic adaptation of ruminants to ensure that glucose and glucogenic compounds are conserved for body processes for which these substances are essential.

1.2.2 Protein and minor nitrogenous fractions

Milk protein is widely consumed as a human food, particularly in developed countries. The long history of it's inclusion in the human diet and the relative ease with which the major proteins can be purified from raw milk have contributed to it's early and extensive characterisation by scientists. Consequently, milk proteins are probably the best characterised of all food proteins (Swaisgood, 1992). Milk proteins are of two distinct types, whey proteins (serum proteins) and caseins. Caseins constitute over 80 % of the total protein of bovine milk although the proportion of whey proteins to caseins varies according to a number of factors, including the stage of lactation (Varnam and Sutherland, 1994).

1.2.2.1 Casein proteins

In milk, caseins are organised into particles with an average size of about 100 nm. The caseins of milk may be subdivided into five main classes, α_{s1}, α_{s2}, β-, γ-, and κ-casein (Table 1.3). Various contrasting models have been proposed for the structure of casein micelles (Banks *et al.*, 1981), but there are some common features. It is recognised that the micellar framework comprises a network of α_s-casein complexed

with calcium phosphate. There is evidence that the serine phosphate ester groups are inherent in the structure of the micellar calcium phosphate. Within the framework of α_s-caseins both the β- and κ-caseins are accommodated. The relative proportions of α, β- and γ-caseins are subject to genetic variation within individual herds and there can be significant differences in casein composition of milk from different cows. The casein composition of bulk milk, however, varies very little at any stage in lactation (Walstra and Jenness, 1984; Swaisgood, 1992; Varnam and Sutherland, 1994).

Table 1.3 The Caseins of milk.

Fraction	**Phosphoserine residues**
Alpha $_{s1}$	7 - 9
Alpha $_{s2}$	10 - 13
Beta	5
Gamma	0 - 1
Kappa	1

(Varnam and Sutherland, 1994)

1.2.2.1.1 Structure of individual casein proteins

The primary structure of the caseins is completely defined (Eigel *et al.*, 1984). Caseins exist in milk in the form of supramolecular aggregates, imparting opalescence to skim milk. Caseins are not present in milk as individual molecular structures, but rather as large protein complexes that also incorporate milk salts, particularly calcium salts (Schmidt, 1980). The 'native' structure is actually a protein complex resulting from interaction of all the individual caseins (Swaisgood, 1992). Because of this property, they cannot be denatured by heating (Early, 1992). The α- and β-caseins are phosphoproteins containing between 5 and 13 phosphoserine groups. These phosphoserine residues are the key to the unique properties of casein. They account for the calcium sensitivity of milk protein. When calcium is added to casein solutions, the casein promptly coagulates as inter-

molecular calcium bridges are formed. Clusters of phosphoserine residues are responsible for the highly charged hydrophilic areas leading to the presence of hydrophilic and hydrophobic domains in a single molecule that allows the proteins to act as highly effective surface active agents, and consequently they have found widespread use as emulsifier agents (Coupland and Mc Clements, 1996). γ-Caseins are the result of post-secretory hydrolysis of β-casein. In good quality milk, γ-caseins constitute a very low proportion (less than 5 %) of the total casein fraction in milk. κ-Casein differs from the other caseins, in that it is insensitive to the addition of calcium, as it has one serine phosphate ester group and contains a charged carbohydrate moiety (Early, 1992).

1.2.2.2 Whey proteins

Whey can be defined in a very general sense as the liquid remaining after removal of casein from milk (Mulvihill and Donovan, 1987). The whey, or serum proteins are compact gobular proteins and include four gene products; β-lactoglobulin, α-lactalbumin, lactoglobulins (immunoglobulins) and bovine serum albumin (Table 1.4).

Table 1.4 The Whey Proteins of Milk.

Fraction	Molecular Weight (kDa)
Beta-lactoglobulin	18.3
Alpha-lactalbumin	14.2
Serum albumin	63.0
Immunoglobulins	up to 1000

(Varnam and Sutherland, 1994)

1.2.2.2.1 β-Lactoglobulin

The major protein constituent of whey is β-lactoglobulin, which is present to the extent of approximately 50 % of the total whey protein of milk and approximately 14 % of the total protein of bovine milk. However, it is absent from human milk. Structurally, it consists of a single polypeptide chain (MW 18.3 kDa) containing 162

amino-acid residues and is usually present as a stable dimer between pH 5.5 and 7.5 (Mc Kenzie, 1971). Thermal denaturation of bovine β-lactoglobulin causing the dimer to dissociate to momomer has been shown *in vitro* when the temperature was increased from 30 to 55° C (Mc Kenzie, 1971). Denaturants include alkali, heat, organic compounds and heavy metal ions (Hambling *et al.*, 1992). A major consequence of denaturation is a reduction in protein solubility, which has been exploited to monitor the heat processing history of milk products. Quantitation of whey protein denaturation by measurement of loss of solubility on saturation with NaCl is used as an index of the heat treatment of milk products (Harland and Ashworth, 1947; Leighton, 1962). Another manifestation of whey protein denaturation is increased side-chain reactivity of the sulphydryl groups of β-lactoglobulin which is reported to be responsible for cooked flavour development (Kinsella, 1985) and antioxidant properties (Taylor and Richardson, 1980).

1.2.2.2.2 α-Lactalbumin

α-Lactalbumin is the second most abundant of the whey proteins in bovine milk and has been physically and chemically characterised (Swaisgood, 1992) (Table 1.4). It is the regulatory component of the enzyme lactose synthetase (Brew and Grobler, 1992). It has been postulated that the secretion of α-lactalbumin in the mammary gland controls the levels of lactose synthesis, and a high correlation has been found between the lactose and α-lactalbumin contents of milks of a number of species. Since lactose is the principal osmotic regulator of milk, its synthesis must be rigidly controlled, and this is probably the physiological role of α-lactalbumin (Morrissey, 1985). It is monomeric, existing as a highly compact, virtually spherical shaped protein with a greater heat stability than that of β-lactoglobulin (Varnam and Sutherland, 1994).

1.2.2.2.3 Immunoglobulins

Immunoglobulins are glycoproteins having high molecular weights and possessing antibody activity (Mulvihill and Donovan, 1987). They occur in only small amounts in normal milk, but in much higher concentration in colostrum. Four distinct classes of immunoglobulins occur in bovine milk, IgM, IgA, IgE and IgG. As a group, the

immunoglobulins are either monomers or polymers of a four-chain molecule consisting of two light (MW 22 kDa) and two heavy (MW 50-70 kDa) chains (Eigel *et al.*, 1984).

1.2.2.2.4 Bovine Serum Albumin

Bovine serum albumin (BSA) prepared from milk is physically and immunologically identical to blood serum albumin. BSA contains 582 residues, has 17 intra-molecular disulphide bonds and one free thiol group, which occurs at residue 34. In the model produced by Brown (1977), the BSA molecule is visualised as having three major domains, each consisting of two large double loops and a small double loop and appears to be in the shape of 3:1 ellipsoid.

1.2.2.3 Economic value of milk protein

Protein accounts for approximately 25 % of the total solids in milk and both its absolute concentration and its concentration relative to the concentration of fat and lactose are important in the manufacture of dairy products (Murphy and O' Mara, 1993). Recently, it has been shown that the presence of genetic variants of k-casein and β-lactoglobulin are associated with increased total protein and casein concentrations and with increased cheese yields (Van den Berg *et al.*, 1991). Therefore, selective breeding may offer more opportunities in this regard. Low protein concentrations cause problems for the processor in terms of the efficiency of product manufacture and in meeting certain minimum protein standards for dairy products. Irish data have shown that Cheddar cheese yield decreased 15 % when milk protein concentration decreased 18 % from 35.5g/kg in October to 29g/kg in February (Murphy and O' Mara, 1993). Low milk protein can also be problematic in the manufacture of dairy products. For example, there are minimum protein concentrations set for the demineralised whey protein used in infant feed formulations while some countries specify a minimum protein for whole milk powder.

1.2.3 Enzymes of bovine milk

Bovine milk contains approximately 60 enzymes, which originate from the mammary gland tissue cells, blood plasma and blood leucocytes. Certain flavour and stability problems in dairy products have stimulated considerable research on enzymes such as lipase, xanthine oxidase and protease (Kitchen, 1985). Plasmin is the major proteinase in milk whose concentration varies according to the extent of leakage of blood components into the milk, being high, for example, in very early or very late lactation milk. High concentrations of plasmin also arise as a result of mastitis (Politis *et al.*, 1992). Milk lipase has received considerable attention in the dairy industry owing to it's potential to produce free fatty acids (FFA) from milk triglycerides, thus causing off-flavours. Lipoprotein lipase (LPL) is the principal lipolytic enzyme in milk (Kitchen, 1985). The enzyme is present in large quantities in fresh milk, but the protective effect of the MFGM means that significant lipolysis due to LPL is rare. However, spontaneous lipolysis involving rapid production of FFA and associated rancid flavour can occasionally occur. The causes are not well understood but probably arise from a combination of factors including diet and stage of lactation (Astrup *et al.*, 1980). A number of oxidoreductase enzymes are also present in milk, including catalase, lactoperoxidase and xanthine oxidase (Kanner and Kinsella, 1983; Spencer and Simon, 1960). Lactoperoxidase catalyses the transfer of oxygen from hydrogen peroxide to other substances such as thiocyanate and is present in high concentrations in milk. Lactoperoxidase has the potential to catalyse oxidation of unsaturated fatty acids leading to the development of oxidised flavours. On the other hand, lactoperoxidase can also act as a powerful bacteriocide which can kill coliforms, *Salmonella* and *Pseudomonads* (Early, 1992). Xanthine oxidase can cause 'off-flavour' production through catalysis of oxidation, but this is not thought to be of importance in most circumstances (Kitchen, 1985). The level of catalase in bovine milk varies with somatic cell count, and thus a measure of it's activity has been used to detect mastitis. Catalase, being a haem-containing protein, may also be involved in lipid oxidation in a manner similar to that of lactoperoxidase. Superoxide dismutase is a common constituent of all microbial, plant and animal cells and has the ability to convert superoxide anions into oxygen

and hydrogen peroxide (Halliwell, 1994). It is located wholly in the skim milk fraction of milk. Alkaline phosphatase is another enzyme present in milk and although it is of no importance with respect to milk stability, it is widely used as an index of adequate heat treatment due to it's inactivation by pasteurisation (Varnam and Sutherland, 1994).

1.2.4 Lactose and other carbohydrates

Lactose is the major carbohydrate in the milk of most mammals and it is generally accepted that non-mammalian sources of lactose are very rare. The lactose content of normal bovine milk is generally in the range 4.4 - 5.2 %, whereas it is higher, approximately 7 % in human milk (Varnam and Sutherland, 1994). Lactose is the major constituent of dried milk, constituting 37 % (w/w) , thus at least some of the properties of lactose will be reflected in the behaviour of dried milk particles (Early, 1992). For example, owing to the high hygroscopicity of lactose, dried milk particles readily adsorb moisture from the surrounding atmosphere. When this happens, the concentration of lactose is diluted so that it's molecules acquire sufficient mobility and space to orientate themselves into a crystal lattice, which leads to clumping and caking. Lactose is used extensively in the food industry in infant foods, dietetic foods, fruit, vegetable and dairy products. It is also added to bakery products such as biscuits to impart a controlled degree of Maillard browning, a reaction considered favourable in certain food products (Varnam and Sutherland, 1994). Adverse reactions may occur in lactase-deficient individuals after ingestion of foods containing lactose (Morrissey, 1985). Ingested lactose is hydrolysed by the enzyme lactase which is found within the brush border of the microvillous area of the jejunal mucosa. If intestinal lactase activity is low, the ingested lactose may not be fully hydrolysed, leading to it's transfer to the intestinal lumen causing abdominal distention, cramps and increased peristalsis (Paige and Davis, 1985).

1.2.5 Minerals

Milk contains all the mineral elements of nutritional significance for the growing young of the species (Table 1.5). Cow's milk is characterised by a high calcium content, three or four times that of human milk and by a very low content of iron

Table 1.5 Mineral and Vitamin Composition of Cow's Milk (Cremin and Power, 1985).

Vitamin	Mean/l	Minerals	Mean/l	Minerals	Mean/l
A (μg)	1000	Sodium (mg)	150	Chromium (μg)	40
D (IU)	400	Potassium (mg)	600	Molybdenum (μg)	8
E (μg)	10, 000	Chloride (mg)	430	Nickel (μg)	25
K (μg)	50	Calcium (mg)	350	Silicon (μg)	700
B_1 (μg)	450	Magnesium (mg)	28	Vanadium (μg)	7
B_2 (μg)	1750	Phosphorus (mg)	145	Selenium (μg)	1270
Niacin (μg)	900	Iron (μg)	760	Vanadium (μg)	7
B_6 (μg)	500	Zinc (μg)	2950	Arsenic (μg)	50
Pantothenic acid (μg)	3500	Copper (μg)	390		
Biotin (μg)	35	Manganese (μg)	12		
Folic acid (μg)	55	Iodine (μg)	70		
B_{12} (μg)	4.5	Fluoride (μg)	77		
C (mg)	20	Selenium (μg)	14		

(Early, 1992). Various nutritional aspects of essential minerals in bovine and/or human milk have been reviewed in recent years (Flynn and Power, 1985; Hambraeus, 1992). The essential minerals are classified into two groups, i.e. the minerals and trace elements. The macrominerals (sodium, potassium, chloride, calcium, magnesium, phosphorus and sulphur) are present in the body in amounts greater than 0.01 % by weight while the trace elements (the remaining 15 essential minerals) occur in the body at much lower levels and are required in the diet in amounts less than about 100 mg/day (Flynn and Power, 1985). The mineral composition of human and bovine milk is not constant but is influenced by a number of factors including stage of lactation, nutritional status of the mother, environmental and genetic factors. The total mineral content of bovine milk (7.3 g/l) is considerably higher than that of mature human milk (2.0 g/l). This is mainly due to the higher concentrations of sodium, potassium, chloride, calcium, phosphorus and magnesium in cows milk (Table 1.5) (Flynn and Power, 1985). A 60 g weight of dried skim milk can provide the recommended dietary allowance (RDA) for calcium and 75 % of the RDA for phosphorus. Whilst dietary calcium has been widely recognised as a key nutritional factor in determining healthy bone development in growing young, deficiency is only cause of osteoporosis in post-menopausal women (Heaney *et al.*, 1982).

1.2.6 Vitamins

Vitamins are organic chemicals required in trace amounts for growth and maintenance of health (Cremin and Power, 1985). Vitamins can be sub-divided into two major groups: fat-soluble and water soluble. Vitamins A, D, E and K are generally found in the lipid fraction of foods and are termed the fat-soluble vitamins. The water soluble vitamins include vitamin C and the vitamins of the B complex: thiamine, riboflavin, niacin, panthothenic acid, vitamin B_{12}, folic acid and biotin (Table 1.5). In metabolism, the water-soluble vitamins and vitamin K function predominantly as coenzymes whereas the remaining fat-soluble vitamins have additional roles in the body. Vitamin A is central to the visual process as a constituent of the visual pigment rhodopsin. Vitamin D is necessary for calcium

absorption, while vitamin E has an antioxidant role. Milk and dairy products contribute significantly to the RDA for many of these vitamins (Cremin and Power, 1985).

The vitamin content of milk (Table 1.5) and dairy products is altered by many factors including animal diet quality, genetics, stage of lactation, season and food processing. Bovine milk typically contains an average of 20-50 ppm vitamin E, but animal diet quality can cause large variations in it's content (McGillivay, 1956). Vitamin E occurs in cow's milk exclusively as α-tocopherol. The concentration of vitamin E in bovine milk exhibits a seasonal pattern reflecting variations in dietary vitamin E intake, with a maximum in April and a minimum in September (Early, 1992; O'Shea 1996 personal communication). Vitamins C, riboflavin and vitamin A are all affected by light to varying degrees. The fat soluble vitamins are relatively stable to heat but the water soluble vitamin are less heat stable. Loss of vitamin C is closely related to the oxygen content of the product and steps should be taken during milk processing to avoid excessive aeration. The nutritional value of milk is often underestimated and arguments about the relative merits of different fat types have tended to obscure the undoubted nutritional benefits associated with the consumption of milk vitamins, minerals and proteins (Early, 1992).

1.3 MILK POWDER

Preservation of the nutritive value of milk, while at the same time maintaining it's flavour quality and extending it's shelf-life can be achieved through spray-drying of milk. By almost complete removal of water from milk, drying processes yield powders which are convenient to transport and use. Whole milk powder is the product obtained by dehydrating whole fluid milk.In countries where the indigenous milk supply is inadequate, whole milk powder is reconstituted for consumption as a substitute for fresh milk and for the manufacture of dairy products. On the domestic European market, whole milk powder is utilised as an ingredient by the food industry for product manufacture (Tuohy, 1987).

1.3.1 Whole Milk Powder Manufacture

Milk powder manufacture is an old process and it is known that the Mongolians produced milk powder by drying whole milk in the sun (Faldt and Bergenstahl 1995). In his writings, Marco Polo reports that in the 12th century, the Tartars produced a milk powder by exposing thin films of milk to the sun. However, it was only in 1875 that Grinwade patented a process for the manufacture of milk powder in the U.K. This process was used in the U.S. by 1887 and in Canada and Europe by the early 1900s (Caric, 1994). From approximately 1930, the spray-drying process started to gain importance and great developments have taken place in recent years. Milk drying has become an essential part of the long chain between the farm producer and the final domestic customer, and dried milk has grown into a large industry of considerable international importance. The diversification of the dry milk industry has been described by Robinson (1986) who outlined the potential use in the food industry of the non-fat milk solids and the whey proteins. The drying of milk takes place in 5 stages; standardisation, heat treatment, evaporation, homogenisation and spray-drying as outlined below.

1.3.1.1 Standardisation

Standardisation, or correction of the fat/non-fat solids ratio, is an adjunct step in the drying process. To achieve the desired ratio in the final product, these components are adjusted before evaporation. Standardisation of milk may be achieved by the removal of fat from whole milk and/or the addition of liquid skimmed milk or cream to whole milk (Tuohy, 1987). For the production of milk powder, it is recommended that the fat/non-fat solids ratio be adjusted to 18-25 %. The fat and non-fat solid contents of milk are analysed and on the basis of these values and the desired ratio in the product, the value to which the fat content should be corrected is calculated by material balance. A mathematical formula called Pearson's Square in used in the standardisation of milk. The Pearson Square method is a simple diagrammatic method of determining the amount of skim milk required to standardise milk for whole milk powder manufacture.

1.3.1.2 Heat Treatment

The whey proteins in milk are particularly sensitive to heat and can easily be denatured at temperatures above 65° C. Hence, whole milk may be given controlled heat treatments in the pre-heat section of the evaporator to achieve desired levels of whey protein denaturation. The degree of whey protein denaturation influences the functional properties of the resultant powder and it's suitability for use in particular applications. Heat treatment is usually carried out at temperatures higher than pasteurization in continual heat exchangers of a plate or tubular type (Caric, 1994). The goals are (i) to destroy all pathogenic bacteria and most of the saprophytic micro-organisms, (ii) to inactivate enzymes, especially lipase, which could provoke lipolysis during storage, (iii) to activate sulphydryl groups of β-lactoglobulin, thus increasing resistance of the powder to oxidative changes during storage and (iv) to achieve a particular heat treatment class (Early, 1992). There are three general heat treatments used in the manufacture of spray-dried whole milk powder. The different heat treatments can be differentiated by determining the undenatured whey protein nitrogen index (ADMI, 1971) (Table 1.6).

Table 1.6 The utilization of low-, medium- and high-heat powder.

Product	Powder Type	Attributes
Cheese	Low heat	Rennetability
Reconstituted milk and milk drinks	Low/ medium heat	High solubility, minimal cooked flavour
Ice cream	Medium heat	Emulsification, foaming, water absorption
Recombined evaporated milk	High heat	Heat stability

(Modified from Varnam and Sutherland, 1994).

(i) Low heat powder is produced by subjecting milk to between 70° C and 75° C, for 10 seconds. The powder obtained, referred to as 'low heat powder', must contain a minimum amount of undenatured whey proteins, that is, greater than or equal to 6.0 mg/g milk powder. Low heat powder has relatively little protein denaturation or

aggregation because it has received minimal heating during manufacture and therefore is ideal for reconstitution into beverages such as skim milk or, more usually, as recombined or filled pasteurised milk. It is also ideal for the manufacture of 'instant' skim milk powder, milk placer formulations, cottage cheese and cultured milks (O' Sullivan and O' Connor, 1971).

(ii) Medium heat powder is produced by subjecting milk to between 76.5° C and 85° C, for 15 to 30 minutes. Consequently, medium heat powder has a greater degree of protein denaturation as a result of the more severe heat treatment than low heat powder. Medium heat powder contains between 1.5 and 6.0 mg of undenatured whey proteins/g milk powder and is generally used in the manufacture of recombined or filled evaporated milk and sweetened condensed milk, as this heat treatment classification is associated with heat stability (O' Sullivan and O' Connor, 1971).

(iii) High heat powder is produced by subjecting milk to between 90° C and 120° C for 2 minutes and consequently possesses a higher degree of denaturation than powders of lower heat classes. Heating at higher temperatures with shorter holding times produces a more soluble and better-tasting powder. High heat powder contains less than 1.5 mg of undenatured whey protein/g of milk powder according to the WPNI index (ADMI, 1971) and is used for baking purposes because very severe heat treatment is necessary to denature the dough depressant factors native to milk (O' Sullivan and O' Connor, 1971).

1.3.1.3 Evaporation

In the manufacture of whole milk powder, the heat-treated milk is subsequently concentrated by evaporation. Concentration by evaporation using falling film evaporators is most commonly used although increasing use is being made of ultrafiltration and to a lesser extent reverse osmosis (Early, 1992). Evaporation is a compulsory step in powder processing for several reasons. Milk powder produced from evaporated milk has longer shelf-life and larger powder particles with a smaller amount of occluded air (Pisecky, 1980). The omission of the concentration stage would not be

economically justified because the drying process would be prolonged, the equipment would not be fully utilized and energy consumption would be markedly increased. Some 80 % of the water in milk is removed by evaporation to produce a concentrate of about 50 % total solids, suitable for spray-drying.

1.3.1.4 Homogenization

Homogenization is conducted after evaporation, or in partly concentrated milk. The main objective of concentrate homogenisation is to reduce the average diameter of the fat globule to < 1 μm (De Vilder *et al.*, 1979) leading to a 4-6 fold increase in fat globule surface area. There is insufficient natural membrane to coat the newly formed surface fully and consequently the globules are coated with a new membrane which consists mostly of casein. The membrane of homogenised milk contains 2.3 g protein/100 g fat which is considerably higher than the level of protein in the natural membrane (0.5-0.8 g protein/100 g fat) (Fox, 1991). Fat globules without protective membranes reduce milk powder solubility and increase susceptibility to oxidative rancidity (Caric, 1994).

1.3.1.5 Spray-Drying

The objective of spray-drying is to convert a liquid into a dry material, thus providing a product with an extended shelf-life (Boersen, 1990). Fig. 1.2 depicts a conventional spray-dryer used in the manufacture of whole milk powder. Spray-drying is an extremely complex process involving a number of key steps. Milk concentrate is first converted into a fog-like mist ('atomised'), whereby it is given a larger surface area. The atomization conditions are central to the spray-drying process, and play an important role in defining such powder properties as particle size, particle density and bulk density through their influence on droplet size (Bloore and Boag, 1982). The atomised liquid is exposed to a flow of hot air in a drying chamber. The air has the function of supplying heat for the evaporation and, in addition, it acts as carrier for the vapour and the powder. When the atomised product is in contact with the hot air, the moisture evaporates quickly and the solids are recovered as a powder consisting of fine, hollow, spherical particles with some occluded air. In single-stage drying the spray dryer is operated in such a way that the powder has reached the final moisture content when leaving the drying chamber whereas in the two-stage drying process, the primary

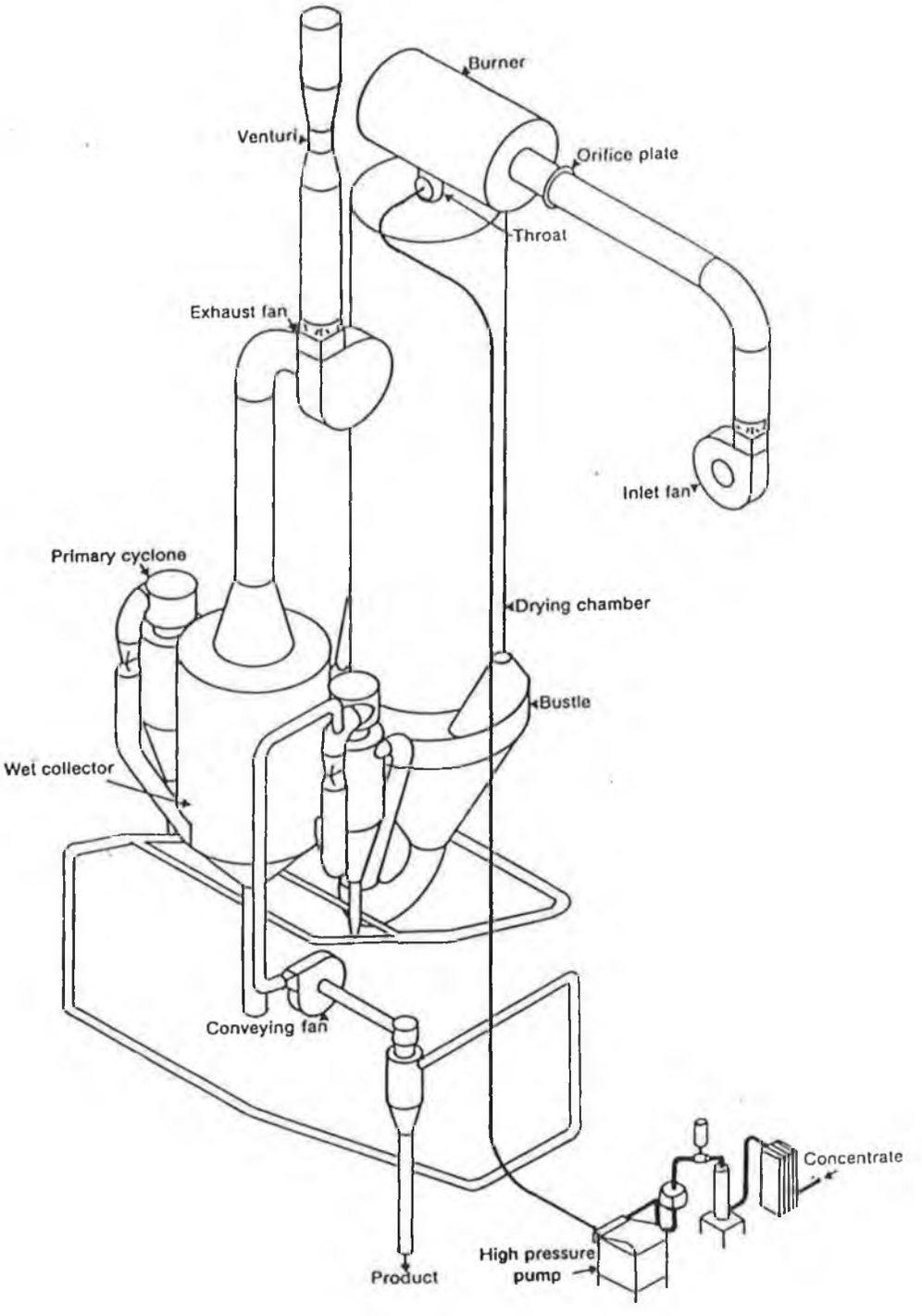


Fig. 1.2 Schematic of a spray-dryer (Boersen, 1990).

drying is carried out to about 2-10 % above the final moisture content. The excess moisture is evaporated in a fluid-bed or vibro-fluidizer, in which air is distributed over the whole milk powder, thereby gently evaporating the rest of the moisture (Boersen, 1990).

1.3.1.6 Packaging and storage of whole milk powder

Packing of milk powder is especially important to retain optimum product quality during storage (Tuohy, 1984). The powder is packed in suitable containers designed to protect the powder from moisture, air, light, insects, and contamination. Wrappings typically used include paper, multilayer boxes or bags with a polyethylene layer inside, metal barrels with polyethylene bags inside and cans covered with aluminium foil on the contact surface. When the product is intended for long-term storage, the packing is performed in an atmosphere of inert gas, mostly nitrogen, or in partial vacuum of 4.0-5.3 kPa, to avoid oxidative changes of fat and other milk components. Investigations of the changes of spray-dried milk powder during storage in an atmosphere of nitrogen and in oxygen at 37° C have shown a decrease in levels of lysine, folate, and ascorbate in the atmosphere of oxygen, while in an atmosphere of nitrogen there was no significant loss of any component (Caric, 1994).

1.3.2 Whole milk powder quality

Properties of milk powders can be categorised as (i) chemical, (ii) microbiological, (iii) physical, (iv) functional and (v) organoleptic (Early, 1992). The quality of milk powders and other dried milk products is defined by quality specifications (ADMI, 1971). Powders are manufactured to specifications which define, in particular, the chemical, microbiological and physical standards. Functional standards may also be cited, but for the most part will relate to performance characteristics of the powder on reconstitution. Functional performance in a given application can be difficult to quantify in terms suitable for a specification. Organoleptic properties are probably the least precisely defined, as they relate only to a typical flavour or absence of taint. The distinction between the categories is not clear cut, e.g the degree of denaturation of whey proteins is relevant to the chemical properties of a powder, but it also influences the functional properties of the milk powder (Early, 1992).

1.3.2.1 Chemical properties

The chemical properties of a milk powder are dependent on the composition and quality of the raw materials used in manufacture and can be influenced by the processing conditions during manufacture, e.g. heat-treatment which can result in Maillard reaction products (Lingnert and Hall, 1986) with consequent browning and flavour development.

1.3.2.2 Microbiological properties

Dried milk, because of it's low water activity, is a stable product and does not support the growth of microorganisms in the dry state, but it can support growth of microorganisms following reconstitution. Any microorganisms present in the dried milk will subsequently multiply in the reconstituted product. The heat treatment given to milk during different stages of drying is severe enough to destroy most pathogens in milk, though some thermophilic organisms can survive, e.g. thermophilic sporeformers. The microbiological properties of a powder are defined in terms of (i) absence of pathogenic organisms; (ii) absence of food spoilage organisms and (iii) a maxima of plant hygiene indicators (Early, 1992).

1.3.2.3 Physical properties

The physical properties of a milk powder are varied and important in influencing the shelf-life of the powder and the handling characteristics. They include (i) moisture, (ii) bulk density, (iii) insolubility index and (iv) scorched particles. Free moisture content is the most important physical property of a powder, with an optimum of 3-4 % being desirable in whole milk powder. Water can influence the chemical reactions in milk powder in various ways (Van Mil and Jans, 1991). It plays a role in controlling the oxidation in foods (Karel, 1980) and in the promotion of Maillard reactions (Lingnert and Hall, 1986). Bulk density is defined as the measure of a given volume of powder, usually expressed as g/cm^3 or kg/l. The importance of bulk density is primarily one of economics, since a high bulk density powder will give benefits in packing, storage and transport. The bulk density of powder is dependent on the interstitial air in the powder and the particle density. The more interstitial air in a powder, the lower the bulk density. By producing a powder with

wide particle size distribution, smaller particles can pack between larger particles, such that the bulk density is increased. This can be achieved by drying from high solids of the concentrate. High particle density is also important in achieving a high bulk density. To achieve a high particle density the incorporation of air into the feed concentrate must be avoided by avoiding agitation of the concentrate prior to drying (Bloore and Boag, 1982). Bulk density should be taken into account when planning the quantity of wrapping used in storage of whole milk powder (Tuohy, 1984). Solubility is important for most applications as milk powders are reconstituted in water and are therefore required to be highly soluble. However, even spray-dried powders will contain a small amount of insoluble material, which may be expressed and measured in terms of an index (ADMI, 1971). Traditionally the term solubility index has been used but, since insoluble material is being measured, insolubility index is more accurate (Early, 1992). Scorched particles consist principally of burned powder deposits within the drying chamber, particularly around the atomiser and air dispenser. Scorched particles are undesirable in milk powders due to the 'burnt' flavour and poor appearance.

1.3.2.4 Functional properties

Spray-dried products are manufactured for the consumer market or for further processing. Depending on their further application, they need to possess a number of functional properties, such as wettability, flowability and whey protein nitrogen index (WPNI) (Boersen, 1990). The latter is one of the most important functional properties of whole milk powder. Before 1967, milk powder manufactured in Ireland was of an undefined heat treatment classification (O'Sullivan and O'Connor, 1971). Since then, however, the Irish milk powder industry has become more sophisticated in it's manufacturing activities, and hence the need for various heat classifications of milk powders. The heat-class of milk powders is defined by the extent of denaturation of the globular heat-labile whey proteins during the pre-heating stage. Thus the degree of heat treatment can be quantified by measuring suitable changes which occur on denaturation of these whey proteins. The standard

method in use is the undenatured whey protein nitrogen index (WPNI) (ADMI, 1971).

1.3.2.5 Organoleptic properties

A major aspect of the quality of whole milk powder is its sensory quality, which may be defined as the colour and flavour of the powder (Van Mil and Jans, 1991). The organoleptic properties of a milk powder are dependent on the composition of the powder, the quality of the raw materials and the effects of processing such as heat treatment and storage. Storage has a significant impact on organoleptic quality and while whole milk powder containing milkfat may have a typically sweet milk flavour at the point of production, after a period of storage rancid flavours may become predominant. The type of packing and storage conditions significantly contribute to reduction/prevention of the rate of flavour changes in many powders (Tuohy, 1987; Van Mil and Jans, 1991; Chan *et al*, 1993). Additionally, milkfat and lactose may easily absorb and adsorb taints, respectively, from the environment, a characteristic which emphasises the importance of impermeable packaging and clean hygienic storage conditions. Severe heat treatments, such as those employed in the manufacture of high-heat powder and heat-stable skimmed milk powder will induce the production of Maillard reaction products, resulting in typical cooked/carmelised flavours (Lingnert and Hall, 1986).

1.3.3 Fat in whole milk powder

Whole milk powder, having a legally defined minimum fat content of 26-28 % is prone to oxidative deterioration and requires sophisticated drying and packing to minimise autoxidation of lipids during storage. Many researchers have observed that only part of the fat in whole milk powder can be extracted by fat solvents under standardised conditions. This fat is usually termed 'free fat' or extractable fat (Buma, 1971a and b) and can be defined as non-globular fat (Fox, 1991). It is usually expressed as % weight of the powder or as % of total fat content. Roller-dried homogenised whole milk powder was reported to have a free fat content as high as 85-95 % of total fat, much of the free fat being located on the surface of the powder particles (Tuohy, 1987) whereas spray-dried homogenised whole milk

powder had free fat levels of only 2.2-7.5 % total fat. Free fat has been reported to be more readily oxidisable than non-extractable fat (Chan, 1992). In milk powders, the lipid phase can be distributed differently in the powder particle; encapsulated inside the powder particles or spread at the particle surface (Buma, 1971 a). Granelli *et al.* (1996) investigated the influence of surface fat on cholesterol oxidation in stored model food systems. They used powders with 75 %, 50 % and 25 % surface fat, and found that the powder with the highest surface fat had the largest level of COPs after 6 months storage. In contrast, on storage, no correlation was found between free fat, defined as solvent extractable fat, and the amount of COPs formed. Although surface fat is a form of free fat, Granelli *et al.* (1996) found no correlation between the actual surface coverage of fat and the free fat. This is consistent with previous observations made by Buma (1971 b) where no correlation between free fat and the development of oxidation flavour in whole milk powder was observed.

1.4 LIPID OXIDATION

Lipid oxidation leading to rancidity has long been recognised as a problem associated with the storage of fats and oils (Gray and Monahan, 1992). Lipid oxidation refers to a series of basic chemical reactions involving polyunsaturated fatty acids and/or cholesterol that occur in bulk fats and oils generally resulting in a deterioration in quality. Lipid peroxidation is the equivalent term found in biochemical and medical literature to describe oxidative deterioration of unsaturated lipids as it occurs in cells and tissues *in vivo* (Halliwell and Chirico, 1993; Rice-Evans and Gopinanthan, 1995). The interest in lipid oxidation in the last 10-15 years can be attributed in large to the accumulating evidence that free radicals and reactive oxygen species participate in tissue injuries and diseases (Halliwell and Chirico, 1993; Halliwell, 1994; Sevanian and Peterson, 1986).

1.4.1 Mechanism of Lipid Oxidation

Several reviews of the chemistry of lipid oxidation have been published in the last decade (Chan, 1987; Grosch, 1987; Frankel, 1991; O' Connor and O'Brien, 1994). It is essentially a free radical chain reaction involving initiation, propagation and termination stages.

A free radical is simply defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Chirico, 1993). Examples of free radicals are superoxide ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), singlet oxygen ($^1O_2$) and nitric oxide ($NO^{\bullet}$). High energy irradiation of aqueous solutions produces highly reactive $OH^{\bullet}$ that can attack all biological molecules, including membrane lipids. The superoxide radical is not very reactive by itself, but its reactivity can be amplified by a number of agents such as nitric oxide, or by interaction of hydrogen peroxide with haem proteins, transition metal ions or myeloperoxidase (Rice-Evans and Gopinathan, 1995). Ground state oxygen may be activated to singlet state oxygen by electron excitation. The oxygen activated to the singlet excited state reacts much more readily with unsaturated lipids than ground state oxygen.

Lipid oxidation is initiated by the abstraction of a hydrogen atom from a methylene group of a polyunsaturated fatty acid.

$$-CH_2- \quad + \quad {}^{\bullet}OH \longrightarrow \quad -{}^{\bullet}CH- \quad + \quad H_2O$$

methylene group *hydroxyl radical* *carbon radical*

This initial H abstraction is defined as *first chain initiation.*

Initiators in food systems may be UV light, transition metal ions and certain enzymes such as xanthine oxidase and lactoperoxidase. The abstraction of the hydrogen leaves behind an unpaired electron on the carbon. This alkyl radical tends to be stabilised by a molecular rearrangement to form a conjugated diene. While these may form cross links with eachother, by far their most likely fate under aerobic conditions is to combine with O_2 to form a lipid peroxy radical (Kanner and Rosenthal, 1992):

$$-{}^{\bullet}CH- \quad + \quad O_2 \longrightarrow \quad -{}^{\bullet}CHOO-$$

carbon radical *peroxy radical*

This reaction with oxygen still remains the object of much research and uncertainty. Some authors suggest that the oxygen must be in an excited singlet state to react readily with the lipid to produce a peroxy radical ($ROO^{\cdot}$). Singlet oxygen may be generated by a number of different pathways as proposed by Korycha-Dahl and Richardson (1980):

1. Chemical reaction between any residual hypochlorite (formed by myeloperoxidase) and hydrogen peroxide in milk.

   $$H_2O_2 + {}^{-}OCl \longrightarrow {}^{1}O_2 + H_2O + Cl^{-}$$

2. Chemical or peroxidase-catalysed reactions involving metalloproteins.

   $$\text{Peroxidase—OCl} + H_2O_2 \longrightarrow {}^{1}O_2$$

3. Photochemical oxidation in the presence of a sensitiser, e.g. riboflavin in milk.
4. Interaction between secondary peroxy radicals.

   $$2\ \text{R- HCOO}^{\cdot} \longrightarrow {}^{1}O_2 + \text{R-HC=O} + \text{R-HCOH}$$

5. Oxidation of superoxide by selected oxidising agents.

Other reactive oxygen species together with active oxygen iron complexes can also react readily with the carbon radical to produce a peroxy radical. These peroxy radicals are capable of abstracting H from another lipid molecule, i.e. an adjacent fatty acid side chain to form a lipid hydroperoxide and another alkyl radical.

$$\underset{\textit{peroxy radical}}{\text{-CHOO}^{\cdot}\text{-}} + \underset{\textit{methylene group}}{CH_2} \longrightarrow \underset{\textit{lipid hydroperoxide}}{CHOOH} + \underset{\textit{carbon radical}}{\text{-}^{\cdot}CH\text{-}}$$

The latter can react with O_2 to form another peroxy radical and so the chain reaction of lipid peroxidation can continue. This is the propagation stage.

Lipid hydroperoxides are the primary products of lipid oxidation. They are colorless, tasteless and odourless. They are relatively stable but decompose rapidly in the presence of heat or in the presence of transition metal ions, via the Fenton reaction, to yield a complex mixture of low molecular weight secondary breakdown

products with distinctive odour and flavour characteristics, including alkanes, alkenes, aldehydes, ketones, alcohols, esters and acids. During the later stages of lipid peroxidation polymerisation takes place resulting in termination:

$$-\dot{C}H- + -\dot{C}H- \longrightarrow -CH{-}CH-$$

2 *carbon radicals* — *cross link of fatty acid molecules*

The chain is terminated by reactions of free radicals to give non radical products. An extremely important and useful route of termination is the reaction of free radicals with antioxidants (Karel, 1980), as outlined below in section 6.4.

1.4.2 Measurement of lipid oxidation products

A variety of factors influence the relative amounts of reaction products formed by lipid oxidation and a wide range of tests, from organoleptic evaluation to physical and chemical methods of various complexities have been suggested (Tuohy, 1987; Kanner and Rosenthal, 1992). Because sensory evaluation is subjective, chemical (e.g. peroxide value and thiobarbituric acid value) and physical (e.g spectrophotometric and chromatographic methods) measurements are also used to measure lipid oxidation (Rossell, 1991). The extent of lipid oxidation can be determined by measuring (i) losses of unsaturated fatty acids, (ii) amounts of primary oxidation products and (iii) levels of secondary oxidation products, such as carbonyls (Halliwell and Chirico, 1993).

1.4.2.1 Measurement of primary oxidation

Methods that measure primary changes may be classified as those that quantify the loss of reactants (unsaturated fatty acids or oxygen) or the formation of primary oxidation products (hydroperoxides) (Karel, 1980; Tuohy, 1987). The most widely used index monitor of primary oxidation is the peroxide value (PV) test, which reflects the total concentration of peroxides and hydroperoxides present in a fat sample (Kanner and Rosenthal, 1992). Lipid hydroperoxides do not themselves contribute to 'off-flavour' but are unstable intermediate compounds in the oxidation pathway. The most common method for measuring PV is based on iodometric

titration (Loftus-Hills and Thiel, 1946) which measures the iodine produced from potassium iodide by peroxides present (Rossell, 1991) (Table 1.7). The PV is reported in units of mEq O_2/kg fat. Other methods such as high performance liquid chromatography (HPLC) may also be used for peroxide measurement (Halliwell and Chirico, 1993). The measurement of peroxides is restricted by the chemical instability of these compounds. Decay of hydroperoxides can be accelerated by a number of factors such as increasing temperature and storage time (Gray, 1978; Kanner and Rosenthal, 1992).

1.4.2.2 Measurement of secondary oxidation

In situations where oxidation occurs at an accelerated rate and primary products rapidly decompose to stable secondary oxidation products, it may be more accurate to measure the secondary products as an index of lipid oxidation which contribute to the rancid and other objectionable flavours (Gray and Monahan, 1992; Kanner and Rosenthal, 1992) (Table 1.7). One of the most commonly applied assays is the thiobarbituric acid (TBA) test which is based on the reaction between secondary lipid oxidation products and TBA to form a pink chromagen (a $[TBA]_2$ - malondialdehyde (MDA) adduct) which absorbs at 530-532 nm (Halliwell and Chirico, 1993). Much of the early data on the TBA test reported results in terms of parts per million of malondialdehyde. However, it is more accurate to report in units of 'thiobarbituric reactive substances' (TBARS), because compounds other than malondialehyde also react with TBA. Csallany *et al.* (1984) noted that the TBA assay can greatly overestimate the malonadialdehyde present (as determined by size exclusion HPLC). The TBA or more correctly TBARS assay cannot differentiate between fatty acids and sterol hydroperoxides or detect many of the secondary breakdown products, including all the commonly studied COPs and many of the secondary and tertiary fatty acid breakdown products (Addis and Warner, 1992). In spite of the many criticisms of it's reproducibility and reliability, however the TBARS test remains one of the most widely used assays for secondary lipid oxidation.

Table 1.7 Methods used to detect and measure lipid peroxidation.

Method	Compound Detected	Comments
Iodine liberation	Lipid peroxides	Lipid peroxides oxidise I^- to I_2 for titration with thiosulphate. Useful for bulk lipids (e.g. foodstuffs) (Loftus-Hills and Thiel, 1946).
Glutathione peroxidase (GPx)	Lipid peroxides	GPx reacts with H_2O_2 and hydroperoxides, oxidising GSH to GSSG. Addition of glutathione reductase and NADPH to reduce GSSG back to GSH results in consumption of NADPH, which can be related to peroxide content. GPx cannot measure peroxides within membranes (Guenzler *et al.*, 1974).
GC-mass spectrometry	Lipid peroxides and aldehydes	Peroxidation products are extracted, reduced (e.g by borohydride) to alcohols, separated by GC and identified by MS (Halliwell and Chirico, 1993).
Spin trapping	Intermediate radicals	Spin traps (e.g phenyl t-butyl nitrone (PBN)) intercept radicals intermediate in the chain reaction (Schaich and Borg, 1980).
Light emission	Excited carbonyls, singlet oxygen	Self reaction of peroxyl radicals can produce excited state carbonyls and singlet oxygen: both species emit light as they decay to the ground state.
TBA test	TBA-reactive material (TBARS)	The test material is heated at low pH with TBA and the resulting pink chromagen is measured by absorbance at 532 nm. Most of the aldehydes that react with TBA are derived from peroxides and unsaturated fatty acids (Tarladgis *et al.*, 1964).
Diene conjugation	Conjugated diene structures	Oxidation of unsaturated fatty acids is accompanied by an increase in UV absorbance at 230-235 nm. Useful for bulk lipids and LDL .

1.4.3 Biological significance of lipid oxidation products

Feeding trials using oxidised fats and oils have provided ample evidence that lipid oxidation products pose significant biological effects (Peng and Taylor, 1984). In support of this, a wide spectrum of adverse health effects have been produced in animals fed highly oxidised oils. These include diarrhoea, loss of appetite, growth retardation, cardiomyopathy and haemolytic anemia (Sanders, 1983). Severely processed oils contain more oxidised products (hydroperoxides and secondary products) than realistic frying conditions (Kubow, 1990). Thus, it is not surprising that the magnitude of these symptoms may be influenced by the degree of processing and on the type of oil and food used. Mildly oxidised oils have been reported to induce altered fatty acid composition of tissue lipids (Yoshida and Kujimoto, 1989), elevated levels of lipid peroxides in chylomicrons (Naruszewicz *et al.*, 1987), inhibition of prostacylclin production (Ross, 1986), elevated rates of thromboxane A_2 production by platelets and high thromboxane:prostacyclin ratios (Giani *et al.*, 1985), suggesting a relationship between diets containing oxidised fats and conditions predisposing to atherosclerosis. Other studies were carried out which showed that linoleic acid hydroperoxide induced endothelial damage and accelerated the uptake of LDL by cultured arterial smooth-muscle cells (Addis and Warner, 1992). Fatty acid hydroperoxides have been shown to accelerate all three phases of atherosclerosis: (i) endothelial injury, (ii) accumulation of plaque and (iii) thrombosis (Yagi, 1988). Higher serum levels of lipid peroxides were observed in animals and patients with atherosclerosis than in those with no clinical evidence of atherosclerosis (Kubow, 1990). Yagi (1988) investigated the possible atherogenic effects of fatty acid hydroperoxides. Using the TBA methodology, he reported that serum peroxide levels increased with age, and that levels in diabetic patients with angiopathy exceeded levels in diabetics without angiopathy. Malondialdehyde, a major secondary product of lipid peroxidation is present in many foods (Siu and Draper, 1978). Since it is a bifunctional aldehyde, it is a very reactive compound and has been found to take part in cross-linking with DNA and proteins (Addis, 1986). Chronic toxicity studies of orally administered malondialdehyde have

revealed dose-dependent pathological changes in liver (Siu and Draper, 1978). The toxic effects of oxidised lipids have been attributed to an imbalance between the oxidative deterioration of ingested oils and the capacity of the antioxidant defense system (Chow, 1979). Many of these symptoms could be prevented by vitamin E or synthetic antioxidants (Kubow, 1990). Vitamin E is mainly involved in scavenging free radicals and stabilising cell membranes by protecting membrane polyunsaturated fatty acids (Rifici and Khachadurian, 1993).

An increased concentration of end products of lipid peroxidation is the evidence most frequently reported for the involvement of free radicals in human disease. However, such evidence is indirect because epidemiological studies that relate lipid oxidation to cancer or cardiovascular disease concern antioxidant status rather than lipid oxidation directly. For example, the World Health Organisation MONICA study (1985-1991), set up to monitor risk factors for coronary heart disease showed that antioxidant potential (plasma levels of vitamin C, beta-carotene, vitamin A, vitamin E and selenium) was higher in areas of low coronary heart disease (CHD) mortality than in areas of high CHD mortality (Gey *et al.*, 1991).

1.5 CHOLESTEROL OXIDATION

Cholesterol, an unsaturated lipid, undergoes oxidation leading to chemically labile hydroperoxides. These peroxides are readily decomposed to form cholesterol oxidation products (COPs). Various food processing and storage treatments can lead to oxidation of cholesterol in the presence of oxygen, heat, light or radiation. Although more than 70 such oxidation products have been identified (Smith, 1981; Smith *et al.* 1981; Smith *et al.*, 1982), only approximately eight have been detected in food (Sander *et al.*, 1989; Paniangvait *et al.*, 1995). Among the major COPs in foodstuffs are 25-hydroxycholesterol, cholestanetriol, hydroxycholesterol derivatives, α- and β-epoxides and 7-ketocholesterol whose presence in the diet may have a number of potentially negative health implications (Emanuel *et al.*, 1991). Several studies have implicated COPs in the pathogenesis atherosclerosis, carcinogenesis, mutagenesis and cytotoxicity (Bosinger *et al.*,1993). Much of the

research regarding cholesterol oxidation in foodstuffs has focused on eggs, cheese and dairy spreads (Tsai and Hudson, 1984; Morgan and Armstrong, 1992; Nielsen *et al.*, 1995). Recently however, a number of studies have reported levels of COPs in whole milk powder (Nourooz-Zadeh and Appelqvist, 1988; Van de Bovenkamp *et al.*, 1988; Sarantinos *et al.*, 1993; Chan *et al.*, 1993; Rose-Sallin *et al.*, 1995). These studies have shown that while no detectable levels of COPs are evident in freshly prepared milk powders, aged products contain varying amounts, the levels of which are dependent on a number of factors, including drying technology and storage conditions. The work of Nourooz-Zadeh and Appelqvist (1988) has shown that while fresh milk powders prepared by spray-drying in a low-medium heat process exhibited no detectable levels of COPs, high-heat powders contained higher levels (2.8 ppm). Although systematic storage studies were not undertaken, results indicated that storage duration and conditions played a significant role in COPs development. While the oxidation of fatty acids can be recognised by off-flavour, the oxidation of cholesterol does not yield any flavour components. Thus, during storage, dry food products especially, such as milk, cheese and egg powders, may develop cholesterol oxides without incurring unacceptable sensory properties (Addis and Park, 1992).

1.5.1 Structure of cholesterol

The chemical structure of cholesterol is shown in Fig. 1.3. It has a polycyclic nucleus with four fused rings, a branched aliphatic side-chain attached to the D ring at C-17 and a hydroxyl group that is attached to C-3 of the A ring. Of relevance to autoxidation are the B ring unsaturation and positions allylic to it and the presence of two tertiary carbons (C-20 and C-25) in the side-chain (Smith, 1981).

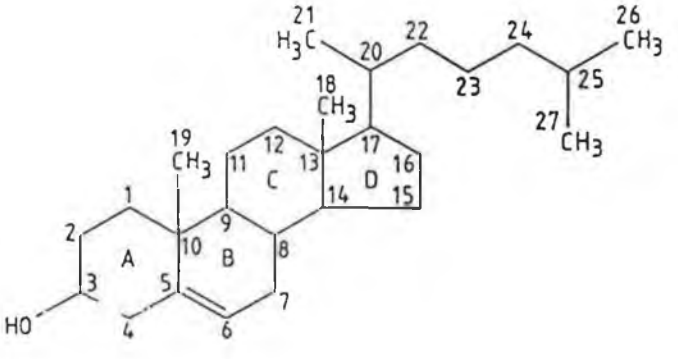


Fig. 1.3 Structure of Cholesterol (Adopted from Nourooz-Zadeh, 1988).

1.5.2 Mechanism of cholesterol oxidation

Cholesterol is an unsaturated compound which readily undergoes autoxidation in the presence of molecular oxygen, light and metal ions through a free radical reaction (Bergstrom and Samuelsson, 1961; Fioriti and Sims, 1967). The initiation process whereby cholesterol reacts with ground state oxygen remains unclear.

Two mechanisms have been proposed: intermolecular oxidation where hydrogen is abstracted from cholesterol by peroxy or oxy radicals of oxidised neighbouring polyunsaturated fatty acids in membranes, or intramolecular oxidation where the oxidised fatty acyl portion attacks the cholesteryl portion of the same cholesteryl ester molecule (Maerker, 1987). In view of the trace amounts of cholesteryl esters in milk lipids, it is probable that the former system pathway predominates during cholesterol oxidation in milk.

Cholesterol oxidation may be initiated by hydrogen abstraction by peroxy or oxy radicals of oxidised neighbouring polyunsaturated fatty acids (Smith, 1987). This is followed by reaction with ground state oxygen ($^3O_2$) and the formation of peroxy radicals as described in Fig 1.4. The abstraction of the H atom predominantly occurs at position C-7. The attack at C-4 seldom takes place due to the influence of the hydroxyl group at C-3 and trialkyl substituted C-5 (Smith, 1980; Maerker, 1987). Subsequently, the chain reaction proceeds when the 7-peroxy radical is stabilised to yield the epimeric cholesterol hydroperoxides. The epimeric cholesterol 7-hydroperoxides are decomposed to the 7α- and β-hydroxycholesterols, respectively (Smith, 1980). Thermal degradation of the epimeric cholesterol 7-hydroperoxides leads to the formation of 7-ketocholesterol.

The cholesterol epoxides are secondary products and are formed from the interaction of cholesterol with sterol hydroperoxides and other oxidising agents (Nourooz-Zadeh, 1988). The ratio of cholesterol-β-epoxide to cholesterol-epoxide formed in solvent and dispersions is governed by the oxidising agent and pH (Smith, 1981).

Hydration of either of the cholesterol epoxides results in formation of cholestanetriol.

Hydrogen abstraction can also take place at the two tertiary carbons in the side-chain, namely at C-20 and C-25 in solid-phase or crystalline cholesterol but is not observed in autoxidations carried out in solution or in aqueous dispersions (Maerker, 1987). Thermal decomposition of the side-chain hydroxyperoxides leads to the corresponding 20α- hydroxy and 25-hydroxycholesterol.

Varying oxidation products of cholesterol are formed, depending on the oxidising agent used. In the presence of the hydroxyl radical ($^{\cdot}OH$), cholesterol undergoes autoxidation through a non-peroxidic reaction yielding certain oxides, namely the isomeric epoxycholesterols, the epimeric hydroxycholesterols and 7-ketocholesterol as the major products (Ansari and Smith, 1979).

Reaction of cholesterol with singlet oxygen ($^1O_2$) occurs via a cyclic ene reaction mechanism with cholesterol α-hydroperoxide as the major product, which is rapidly decomposed to the corresponding hydroxycholesterol (Smith *et al.*, 1973). Table 1.8 summarises the major COPs found in food by both their common and systematic names.

1.5.3 Measurement of COPs

The presence of COPs (oxysterols) in food has been of interest during the last decade as a result of an increasing concern about a possible role for oxysterols in the initiation of atherosclerosis (Peng and Taylor, 1984; Steinberg *et al.*, 1989) and cancer (Addis and Warner, 1992; Rice-Evans, 1994). Based on a number of studies (Nourooz-Zadeh and Appelqvist, 1988; Van de Bovenkamp *et al.*, 1988; Sarantinos *et al.*, 1993; Chan *et al.*, 1993; Rose-Sallin *et al.*, 1995) the most abundant COPs found in aged whole milk powder are the 7-hydroxy derivatives, the 7-keto derivatives and the epoxides. There are, however, large discrepancies among the reported values for COPs in these products (Table 1.9). Variations in temperature,

light and oxygen availability during sample handling may contribute to artefact formation (Smith, 1981).

Table 1.8 Common and Systematic Names of Common Oxysterols Found in Food.

Common Name	Systematic Name
Cholesterol	Cholest-5-en-3β-ol
7α-hydroxycholesterol	Cholest-5-ene-3β-7α-diol
7β-hydroxycholesterol	Cholest-5-ene-3β-7β-diol
19-hydroxycholesterol	Cholest-5-ene-3β-19-diol
25-hydroxycholesterol	Cholest-5-ene-3β-25-diol
Cholestanetriol	Cholestane-3β-5α-6β-triol
Cholesterol-α-epoxide	5,6-α-epoxy-5α-cholestane-3β-ol
Cholesterol-β-epoxide	5,6-β-epoxy-5α-cholestane-3β-ol
7-Ketocholesterol	3β-hydroxy-cholest-5-en-7-one

Table 1.9 Typical levels of COPs found in a variety of milk powders.

Powder Type	Heat Class	Analytical Method	Total COPs (ppm)	Reference
Whole milk powder (fresh)	High	GC-MS*	2.8	Nourooz-Zadeh and Appelqvist, (1988)
Skim milk powder (fresh)	Low	GC-MS	N.D.	Nourooz-Zadeh and Appelqvist, (1988)
Whole milk powder (stored)	Low	GC-FID**	200 ppm	Chan *et al.* (1993)
Full fat cream powder (stored)	Unknown	GC-MS	4 ppm	Rose-Sallin *et al.* (1995)

* GC-MS: Gas Chromatography with Mass Spectrum Detection

**GC-FID: Gas Chromatography with Flame Ionisation Detection

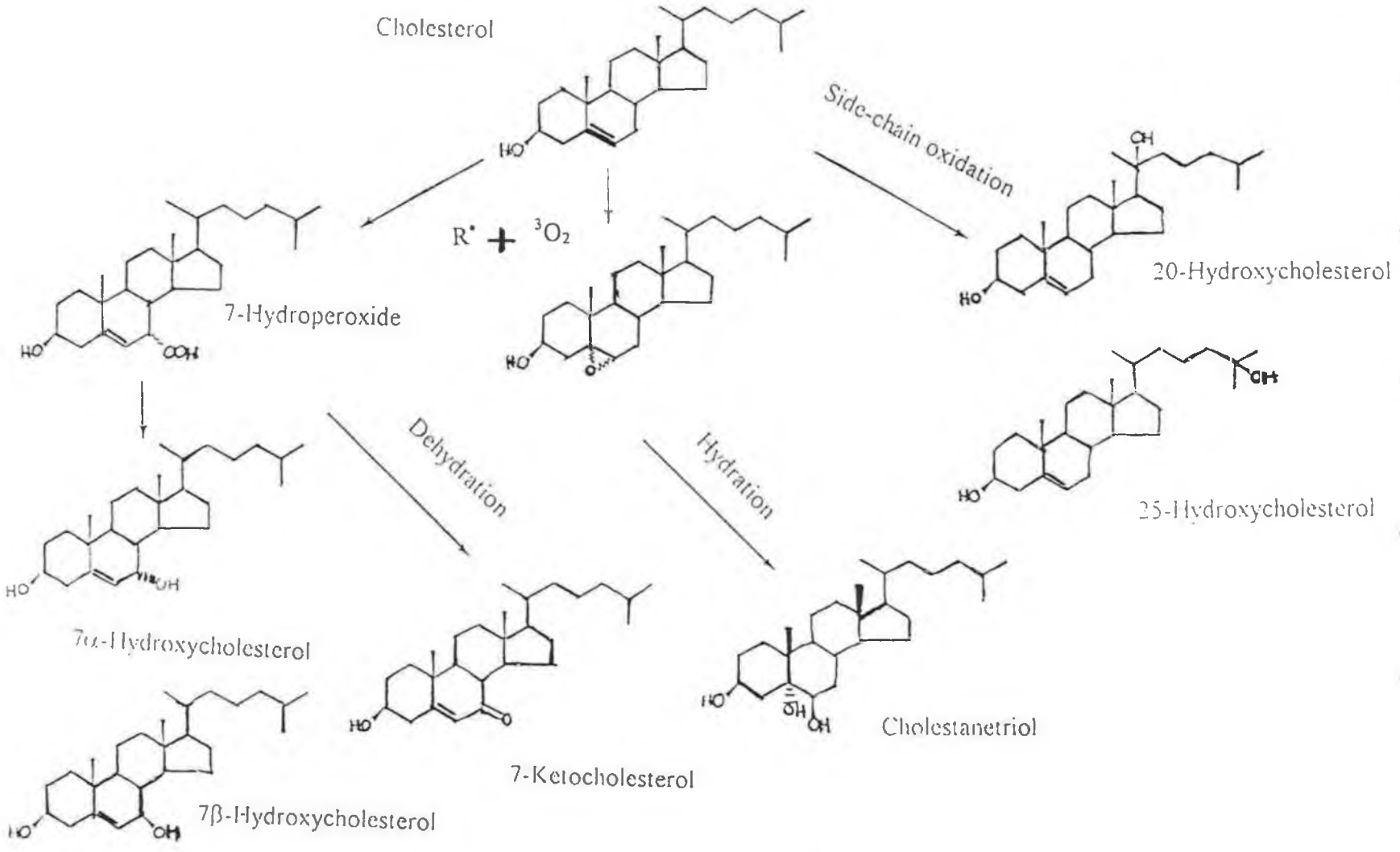


Fig. 1.4 Autoxidation pathways of cholesterol (Paniangvait *et al.*, 1995).

In addition, stability of COPs is greatly influenced by pH (Kim and Nawar, 1991). A variety of analytical methods are in use including HPLC with UV detection (Ansari and Smith, 1979; Sallin *et al.*, 1993) and capillary GC (Park and Addis, 1985a), which in conjunction with GC/MS is a powerful approach for identification/confirmation (Rose-Sallin *et al.*, 1995) at low limits of detection with high specificity (Nielsen *et al.*, 1995). In view of the potential negative health impact of oxysterols from foods, any discrepancies in the levels of COPs reported in food arising from methodology is of major concern with regard to consumer health. A standardized method for quantification of oxysterols has yet to be established (Appelqvist, 1996).

1.5.4 Biological significance of COPs

There is growing interest and concern regarding the potential health implications of COPs in the diet. The effects in humans of chronic ingestion of normal dietary levels of these products are not known (Emanuel *et al.*, 1991; Addis and Warner, 1992). Research on possible postprandial absorption of many specific lipid oxidation products is extremely limited. Bascoul *et al.* (1986) reported that dietary cholesterol α-epoxide was absorbed by the rat and Emanuel *et al.* (1991) has found COPs in human plasma after ingestion of COPs. However, several studies performed both in animal models (Erickson *et al.*, 1977; Jurgens *et al.*, 1987) and in isolated cellular systems (Kandutch and Chen, 1977; Baranowski *et al.*, 1982; Clare *et al.*, 1995) have demonstrated the potential of COPs to produce adverse effects. Several studies have implicated COPs, in particular 25-hydroxycholesterol and cholestanetriol, as the cause of atherosclerotic lesions (Imai *et al.*, 1976; Taylor *et al.*, 1979). α- Epoxycholesterol has been reported to be mutagenic and cytotoxic *in vitro* (Sevanian and Peterson, 1986) and has been detected in UV- induced skin tumours in experimental animals (Black and Douglas, 1973). COPs have direct toxic effects on membrane structure and function by their ability to displace cholesterol (Peng *et al.*, 1979) and thereby alter membrane fluidity (Santillan *et al.*, 1982). Recent studies on smooth muscle cells *in vitro* have shown that cholestanetriol can displace cholesterol from the cell membrane which may explain

it's cytotoxic nature (Mahfouz *et al.*, 1995). Because atherosclerosis has long been directly related to cholesterol (Steinberg *et al.*, 1989), the rest of this section will discuss the current status of the involvement of oxysterols in atherosclerosis. Studies implicating toxicological activities of oxysterols will be discussed in Chapter 6.

1.5.4.1 Effects of COPs on atherosclerosis

Literature on atherosclerosis is dominated by two compelling hypotheses: the 'response to injury hypothesis' and the 'lipid hypothesis' (Addis and Warner, 1992). The response to injury hypothesis proposes that the disease is essentially a response of the vessel wall to injury and although it plays an important role in lesion progression, the deposition of lipid is a secondary phenomenon (Addis and Park 1989). The lipid hypothesis, on the other hand, suggests that atherosclerosis is caused by hypercholesterolaemia-induced deposition of lipid in vessel walls. It implicates cholesterol and not oxidised cholesterol or other oxidised lipids. The atherogenicity of cholesterol is believed to be due to the contaminating COPs and not cholesterol *per se.* Pure cholesterol, in spite of being able to induce hypercholesterolaemia has not been found to be atherogenic or angiotoxic, even in a sensitive animal such as the rabbit (Taylor *et al*, 1979) or in *in vitro* model systems (Clare *et al.*, 1995). On the other hand, small amounts (10-250 ppm) of COPs have shown significant toxicity to many cell lines including vascular endothelial and smooth muscle cells (Peng *et al.*, 1978; 1979). Although dietary cholesterol has long been considered a contributory factor to atherosclerosis in humans (Addis and Park, 1989), more recent studies have indicated a possible role of COPs in the initiation of atherosclerotic plaque formation (Kumar and Singhal, 1991).

Several possibilities exist with regard to the mechanism of oxysterol-induced arterial endothelial damage. Firstly COPs are far more inhibitory to cholesterol biosynthesis than cholesterol itself (Kandutch and Chen 1977, Clare *et al.*, 1995). Inhibition of the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, may render cells deficient in cholesterol, a key

cellular component (Smith and Johnson, 1989), thereby exerting cellular injury (Addis and Park, 1989). Other biological activities of COPs possibly relevant to atherosclerosis include the ability of COPs to inhibit 5'-nucleotidase activity (Peng *et al.*, 1985), thereby inhibiting the ability of endothelial cells to replicate and enable cell repair.

Epidemiological evidence also exists showing that oxidised cholesterol may be related to coronary heart disease. Jacobson (1987) noted that Indian immigrants in London and Trinidad had a 1.5-2.0 times higher rate of CHD than average, a rate which cannot be explained on the basis of accepted risk factors. Both populations consume clarified butter oil ('ghee') in cooking. Jacobson (1987) noted there were substantial amounts of COPs in ghee, but not in fresh butter. A number of studies have been reported that give support to the concept that COPs (in particular triol, epoxide, 25-OH and 7-hydroxyderivatives) are injurious agents capable of initiating atherosclerosis. Calcium accumulation in the aorta of white carneau pigeons has been reported to be involved in the pathogenesis of atherosclerosis (Jacobson *et al.*, 1985). Calcium can accumulate as a result of induced membrane disruption by COPs which disturbs normal ionic homeostasis. Krut (1982) demonstrated that 7α-, 7β- and 25-hydroxycholesterol were effective in solubilizing cholesterol, indicating a possible role for oxysterols in inhibiting atherosclerosis by preventing the crystallisation of cholesterol in tissues. However, the majority of studies in this area of research suggest that COPs are more atherogenic than their native sterol counterpart.

Carpenter *et al.* (1993) analysed lipids and oxidised lipids in samples of human atheroma (necrotic gruel from the interior of advanced atherosclerotic plaques in the aorta) and normal human aorta by GC and GC-MS, and detected significantly higher amounts of 26-hydroxycholesterol and 7α-hydroxycholesterol in all atheroma relative to normal aorta. It is well established that LDL plays a pivotal role in the progression of atherosclerosis, primarily by promoting the accumulation of lipid in the lesion (Steinberg *et al.*, 1989). LDLs are a metabolic end point for apo B-

containing lipoproteins and circulate within the vascular compartment, including the sub endothelial space until removed by high affinity apo B receptor mediated endocytosis. Despite the association between hypercholesterolaemia and atherosclerosis, rarely have researchers identified the involvement of pure cholesterol in the etiological process. Rather, oxidised cholesterol, principally in LDL has been implicated in the etiology of atherosclerosis (Steinberg *et al.*, 1989; Regnstrom *et al.*, 1992). Oxidised cholesterol in LDL may be dietary in origin, or as a result of *in vivo* metabolism and/or radical-induced peroxidation. Dietary sources of COPs may include ingestion of aged whole milk powder (Nourooz-Zadah and Appelquist, 1988; Rose-Salin *et al.*, 1995), dried egg powder (Chan *et al.*, 1993) and deep-fat fried foods (Nourooz-Zadeh, 1988). In vivo metabolism may also result in COPs, for example, the 7α-hydroxycholesterol formed in and secreted from liver (in bile acid biosynthesis) may also be formed in human plasma by leucocytes which possess a cholesterol 7α-hydroxylase (Sobel, 1953). COPs may also arise due to peroxidation *in vivo* by reactive oxygen species (Halliwell and Chirico, 1993). Exposure of LDL to reactive oxygen species chemically damages LDL creating lipid peroxides and ultimately protein adducts of apo B. Oxidative modification of apo B alters its ligand properties (specifically the lysine residue) and marks it for removal by scavenger receptors. Moreover compelling evidence gathered by Walzem *et al.* (1995) that older plasma lipoproteins are more susceptible to oxidation provides a linking mechanism for the lipid and oxidation theories of atherosclerosis. Although the precise mechanism of LDL oxidation in the arterial wall is not known, there is abundant *in vitro* evidence for oxidation of LDL by cells present in the vasculature such as endothelial cells, smooth muscle cells and monocyte/macrophages. A recent study carried out by Chisolm *et al.* (1994) using MS and NMR identified the principal toxin in oxidised LDL from human atherosclerotic lesions as 7β-hydroperoxycholest-5-en-3β-ol. It has been shown to be an intermediate in the non-enzymatic oxidation of cholesterol that leads to the formation of 7β-hydroxycholesterol, 7α-hydroxycholesterol and 7-ketocholesterol. This molecule accounted for approximately 90 % of the cytotoxicity of the lipids of oxidised LDL.

1.5.4.2 *In vitro* cytotoxicity

Cytotoxicity of COPs to arterial smooth muscle cells and endothelial cells appear to have significant implications since these two cell types play major roles in the development of atherosclerotic lesions. Imai *et al.* (1976) studied the effects of autoxidation products of cholesterol which were identified and separated from U.S.P. (United States Pharmaceutical) grade cholesterol which had been stored at room temperature in the presence of air for 5 years. The cytoxicity of these autoxidation products was tested using rabbits as model systems. Aortic examination after 24 hours showed significant increases of degenerative and necrotic arterial smooth muscle cells, whilst the aortas of the animals fed purified cholesterol showed no significant differences from the controls. The minimum concentration in culture medium of the most toxic compounds required to induce aortic smooth muscle cell death within 24 h was reported to be between 0.5 - 1 mg/dl which is approximately one two-hundredth of the normal human serum cholesterol concentration of 150-200 mg/dl (Peng and Taylor, 1984) suggesting that, if only 0.5 % of the serum cholesterol is found to be oxidised, the aortic smooth muscle cells could be injured within 24 hours (Peng and Taylor, 1984). Increased acid phosphatase activity, indicative of cell damage, was observed when human aortic smooth muscle cells were incubated with different concentrations of 25-hydroxycholesterol, cholesterol 5,6-epoxide or 7-ketocholesterol. Since acid phosphatase is a lysosomal enzyme, it's presence is indicative of cell damage (Wolinsky *et al.*, 1975). The most toxic COP, 25-hydroxycholesterol, resulted in the greatest release of acid phosphatase, and the least toxic, 7-ketocholesterol, showed statistically significant release at only the highest level examined (10 μg/ml). It has been demonstrated both histochemically and biochemically that acid phosphatase activity is significantly increased in atherosclerotic lesions in humans and animal models (Wolinsky *et al.*, 1975). Clare *et al.* (1995) found altered mitochondrial membrane integrity and inhibition of HMG-CoA reductase in cultured human monocyte-macrophages on administration of COPs. They found that cholesterol was not toxic and actually inhibited the toxicity of 25-hydroxycholesterol. Lizard *et al.* (1996) found necrosis of bovine aortic endothelial cells as a result of exposure to

COPs. It was shown that 7-ketocholesterol was the most toxic by inducing apoptosis in the endothelial cells treated.

1.6 PREVENTION OF LIPID OXIDATION

Lipid oxidation is of great concern to the food industry because it leads to the development of undesirable 'off flavours' and potentially toxic reaction products. Food manufacturers are therefore concerned with the development of methods for preventing and/or retarding lipid oxidation in foods. To do this effectively, it is necessary to have a thorough understanding of the mechanisms of lipid oxidation (Coupland and McClements, 1996). Prevention of both cholesterol and lipid oxidation in food (Pangiavait *et al.*, 1995) can be achieved by control of dietary, processing and storage conditions. Methods that have been employed to this end include feeding antioxidants to live animals before processing (Wahle *et al.*, 1993; Monahan *et al*, 1990; Li *et al.*, 1996), incorporation of antioxidants prior to processing (Huber *et al.*, 1995), low temperature or minimum temperature processing (Tuohy and Kelly, 1989; Chan *et al*, 1993), packaging with exclusion of O_2 (Chan *et al*, 1993) and low temperature and exclusion of light during storage (Luby *et al.*, 1986).

In view of the many uses for whole milk powder in the manufacture of food products and it's value as an export commodity, it is imperative that the oxidative stability of milk lipids is preserved during manufacturing and storage processes. A number of studies have shown that the process of autoxidation in milk can be minimised or excluded by the use of antioxidants, controlled processing, packing under nitrogen and controlled storage conditions as discussed below.

1.6.1 Processing effects

Storage stability of dried milk is influenced by the pre-heating conditions used (Baldwin and Ackland, 1991; Van Mil and Jans, 1991) with higher pre-heating conditions leading to improved storage stability. Sulphydryl (SH) and disulphide (SS) groups have been widely implicated as important functional groups in many

food proteins (Beveridge *et al.*, 1974). Reactive or free SH groups increase with increased temperature and pre-heating time (Taylor and Richardson, 1980). The production of SH groups, primarily from β-lactoglobulin is of particular importance in whole milk powder as it has been shown to decrease the rate of autoxidation of fat by reaction with free radicals of the unsaturated fatty acids (Van Mil and Jans, 1991). Heat treatment of milk has been shown to increase antioxidant activity with increased 'reactive' sulphydryl groups but decreased 'total' sulphydryl content (Taylor and Richardson, 1980). The antioxidant activity of milk has been shown to reside in both whey and casein proteins with the casein fraction providing the larger portion of antioxidant activity, even though it contains no sulphydryl groups. Thus the sulphydryl groups are apparently responsible for only part of the antioxidant activity of milk (Taylor and Richardson, 1980). Caseins have been reported to be the major protein in milk possessing antioxidant activity (Allen and Wrieden, 1982; Coupland and McClements, 1996). This antioxidant activity may be related in part to their hydrophobic nature and orientation of side chains of constituent amino acids at the lipid interface (Taylor and Richardson, 1980, Ericksson, 1982). Allen and Wrieden (1982) reported that the antioxidant activity of casein derives from its ability to bind metals to its phosphoseryl residues. The binding of metals generally inhibits their catalytic effect on autoxidation.

The rate of lipid autoxidation in air is highly temperature-dependent, the reaction rate has been shown to double with a 10° C rise in temperature (Walstra and Jenness, 1984; Tuohy, 1987; Chan *et al.*, 1993). In parallel, higher temperatures also favour the Maillard reaction, some products of which have antioxidant activity (Lingnert and Hall, 1986; Varnam and Sutherland, 1994). Various Maillard products formed through the interaction of proteins and carbohydrates during the heat-processing of foods are reported to retard the development of rancidity (Baldwin and Ackland, 1991). The formation of brown pigments was first observed by the French chemist, Louis Maillard (1912), following the heating of a solution of glucose and lysine. This reaction was subsequently referred to as the Maillard reaction and essentially covers all those reactions involving compounds with amino groups and carbonyl

groups present in foods. These include amines, amino acids, and proteins interacting with sugars, aldehydes, and ketones, as well as the products of lipid oxidation (Eskin, 1990).

1.6.2 Control of oxygen

Lipid oxidation reactions can be retarded by reducing the concentration of oxygen in the food, for example by packing foods under vacuum or nitrogen. Tamsma *et al.* (1961) reported a statistically significant ($p < 0.05$) improvement in storage stability of whole milk powders packed in inert gases containing 0.1 % oxygen compared with those packed at 1 % oxygen level and that oxygen was depleted to less than 0.001 % within 24 hours in packed milk powder using a scavenging system of 95 % N_2, 5 % H_2 and a platinum catalyst (Tamsma *et al.*, 1967). Abbot and Waite (1962) reported that a mixture of 90 % N_2 and 10 % H_2 in the presence of palladium catalysed the formation of water from the H_2 and residual oxygen to produce an almost oxygen-free atmosphere in the pack. Chan *et al.* (1993) observed that oxygen absorbers effectively controlled lipid and cholesterol oxidation over 6 months storage at 20° C.

1.6.3 Control of Storage Temperature

Higher storage temperatures have led to increased levels of oxidation in milk powders (Tuohy, 1987; Chan *et al.*, 1993, Huber *et al.*, 1995). Boon (1976) subjected whole milk powders to storage temperatures of 22° C and 37° C and found that a significantly greater oxidative deterioration was noted at 37° C compared with 22° C. Tuohy and Kelly (1981) found only a very slight decrease in the quality of whole milk powder stored at 20° C compared to 12° C, whereas they observed significantly greater TBARS values after 24 weeks of storage at 37° C compared to 12° C. Similarly Chan *et al.* (1993) found a marked increase in TBARS and COPs levels in whole milk powder when stored at 40° C compared to storage at 20° C.

1.6.4 Antioxidants

Antioxidants are defined by the United States (U.S.) Food and Drug Administration (FDA) as substances used to preserve food by retarding deterioration, rancidity, or

discoloration due to oxidation (Dziezak, 1986). Their inhibitory effect has been attributed to their donation of electrons or hydrogen to a fat containing a free radical and to the formation of a complex between the antioxidant and the fat molecule (Stuckey, 1972). No single antioxidant offers the definitive answer to oxidative deterioration for all food products. The selection of the appropriate antioxidant appears to be determined by compatibility with and resulting effectiveness in certain fats, i.e. it's solubility in the fat or water phase of the product, good dispersibility throughout the food and it's 'carry through' potential post processing (Dziezak, 1986). Important antioxidants used in foods include the tocopherols, as well as the synthetic antioxidants, butylated hydroxy anisol (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) (Karel, 1980). However, addition of antioxidants to whole milk during processing is not permitted in Ireland (O' Shea, 1996, personal communication). Therefore the most favourable way of increasing antioxidants in food is by dietary means. This has previously been carried out in milk (Dunkley *et al.*, 1967; King, 1968); pork (Monahan *et al.*, 1990; 1992) and eggs (Li *et al.*, 1996) .

1.6.4.1 Vitamin C

Vitamin C (ascorbic acid) occurs widely in plant tissues. The role of vitamin C in mammalian physiology and biochemistry is far from fully understood (Coultate, 1984). It has been implicated in a number of reactions, particularly in the formation of hydroxylysine from the parent amino acids after their incorporation into the polypeptide chain of collagen (Coultate, 1984). Vitamin C, being a strong reducing agent, also functions as a free radical scavenger and in the regeneration of α-tocopherol (Halliwell, 1994). In fruit and vegetable products, notably dehydrated potato, it is used as an antioxidant to prevent the browning reactions that would be catalysed by phenolase. In cured meat products, it is used as a reducing agent to lower the concentration of nitrite needed and is also used as a flour improver (Coultate, 1984).

1.6.4.2 Vitamin E

Vitamin E, the generic term for a group of eight soluble substances, four *tocopherols* and four *tocotrienols* was discovered by Evans and Bishop (1922). The name 'tocopherol' comes from the Greek word 'tocos' meaning childbirth and the Greek verb 'phero' meaning to bring forth (Shukla, 1990). The members of each of the *tocopherols* and *tocotrienols* are designated α, β, γ and δ, depending on the number and position of the methyl groups attached to the chromanol ring. Fig 1.5 shows the structure and properties of the tocopherols and tocotrienols. The side chain is saturated in the tocols and unsaturated in the tocotrienols (Schuler, 1990). The chromanol rings of α-tocopherol and α-tocotrienol molecules fit like pieces of a jigsaw into cell membranes, leaving the phytol chains free. Tocols with differently methylated chromanol rings do not fit into the available space. Hence only α-tocopherol and α-tocotrienol molecules have antioxidant properties *in vivo* (Rice and McMurray, 1983). The most important structural feature of α-tocopherol is the hydroxyl group and three methyl groups on the chromanol ring which confer on the molecule its activity as a biological antioxidant by allowing it to fit into the cell membrane (Rice and Kennedy, 1986). Use of the rat fetal resorption assay for vitamin E has established that the relative order of bioactivity of the tocopherols is α >β > γ > δ (Bunyan *et al.*, 1961). The tocotrienols are much less abundant and, of these, only the α- and β- tocotrienols have been tested for antioxidant properties. The α- form is the most active, but it is substantially less active than it's tocopherol counterpart (Burton, 1994). The structure of the α-tocopherol molecule renders it a highly effective chain breaking antioxidant that readily donates the hydrogen atom from the hydroxyl group to free radicals which then become unreactive.

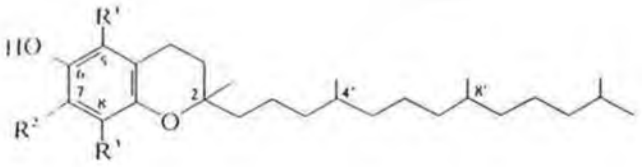
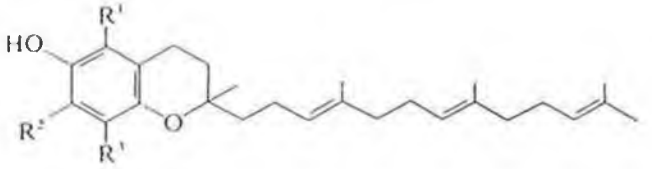
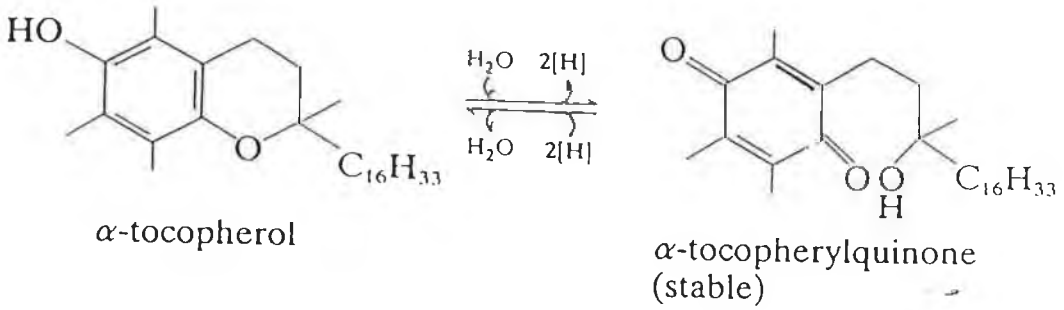
Compound	*Formula mol wt.*	*Structure*
Tocols		
		
Tocol	$C_{26}H_{44}O_2$ 388·64	R^1: H R^2: H R^3: H
8-Methyltocol (δ-Tocopherol)	$C_{27}H_{46}O_2$ 402·67	R^1: H R^2: H R^3: CH_3
5,8-Dimethyltocol (β-Tocopherol)	$C_{28}H_{48}O_2$ 416·69	R^1: CH_3 R^2: H R^3: CH_3
7,8-Dimethyltocol (γ-Tocopherol)	$C_{28}H_{48}O_2$ 416·69	R^1: H R^2: CH_3 R^3: CH_3
5,7,8-Trimethyltocol (α-Tocopherol)	$C_{29}H_{50}O_2$ 430·72	R^1: CH_3 R^2: CH_3 R^3: CH_3
Tocotrienols		
		
8-Methyltocotrienol (δ-Tocotrienol)	$C_{27}H_{40}O_2$ 396·62	R^1: H R^2: H R^3: CH_3
5,8-Dimethyltocotrienol (β-Tocotrienol)	$C_{28}H_{42}O_2$ 410·65	R^1: CH_3 R^2: H R^3: CH_3
7,8-Dimethyltocotrienol (γ-Tocotrienol)	$C_{28}H_{42}O_2$ 410·65	R^1: H R^2: CH_3 R^3: CH_3
5,7,8-Trimethyltocotrienol (α-Tocotrienol)	$C_{29}H_{44}O_2$ 424·67	R^1: CH_3 R^2: CH_3 R^3: CH_3

Fig. 1.5 Structure and properties of Tocols and Tocotrienols (Adopted from Schuler, 1990).

1.6.4.2.1 Mechanism of action

α-Tocopherol (AH_2) functions as a chain-breaking antioxidant. The antioxidant activity of α-tocopherol is mainly based on the 'tocopherol-tocopherylquinone redox system'(Fig. 1.6).



α-tocopherol

α-tocopherylquinone (stable)

Fig. 1.6 α-Tocopherol-tocopherylquinone redox system (Adopted from Schuler, 1990).

α-Tocopherol (AH_2) transfers its H atom to a lipid peroxy radical ($R^{\bullet}$), converting it to a regenerated molecule (RH). The reaction of α-tocopherol with a peroxy radical is faster than the reaction of a peroxy radical with another polyunsaturated fatty acid group. Thus a small amount of α-tocopherol can protect a large amount of polyunsaturated lipid (Burton, 1994).

$R^{\bullet} + AH_2 \longrightarrow RH + AH^{\bullet}$

peroxy radical regenerated lipid

Eventually, the tocopheryl semiquinone radical ($AH^{\bullet}$) may react with another tocopheryl semiquinone radical to form one molecule of tocopherylquinone (A) and a regenerated molecule of tocopherol (AH_2).

$AH^{\bullet} + AH^{\bullet} \longrightarrow A + AH_2$

Each molecule of α-tocopherol can scavenge only two radicals before undergoing regeneration. Therefore, at times of high oxidative stress, the local supply may be exhausted. Fortunately, the radicals are readily reduced to α-tocopherol by ascorbic acid (vitamin C) or other aqueous oxidants (Karel, 1980; Duthie, 1991) as shown below in Fig. 1.7.

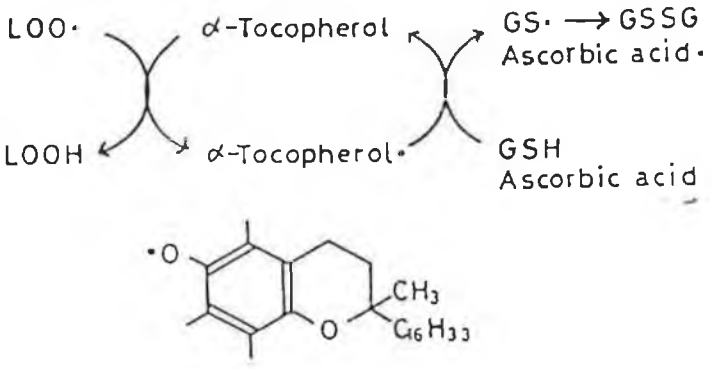


Fig. 1.7 Regeneration of α-Tocopherol by Ascorbic acid (Adopted from Schuler, 1990).

1.7 CONCLUSIONS

Evidence has emerged in recent years that dietary lipid oxidation products (lipid peroxides, malondialdehyde and COPs) may be quite injurious to health. The aim of the work outlined in this thesis was to determine the influence of animal feeding regimen and processing on lipid and cholesterol oxidation in stored whole milk powders. In particular, the effects of pre-heating, packing and storage conditions on primary and secondary oxidation products of lipid and cholesterol were investigated in whole milk powder derived from dietary restricted and supplemented animals. In addition, an *in vitro* system was set up to monitor the effects of COPs on biomarkers of cell function and integrity. This thesis contains four main sections as follows: the first section investigates the development of a method for COPs analysis in whole milk powders (Chapter 2); the second and third sections deal with the nutritional and processing factors that affect lipid and cholesterol oxidation in whole

milk powders (Chapters 3 and 4) and the fourth section of the study describes the effects of COPs found in cell culture studies (Chapter 5).

1.8 AIMS

To evaluate the effects of animal feeding regimen and processing technology on the oxidative stability of lipids and cholesterol in fresh and stored whole milk powders. The second stage of this work was to evaluate the effects of COPs on biomarkers of cell function and integrity.

1.9 OBJECTIVES

- To establish and validate a GC method for the accurate detection and quantification of cholesterol oxidation products (COPs) in whole milk powder.

- To evaluate the effect of supplementary concentrate feeding on lipid oxidation in whole milk powder.

- To assess the effects of processing, packing and storage conditions and the use of natural antioxidants on lipid and cholesterol oxidation and on the flavour stability of whole milk powder during storage.

- To investigate the toxicity of COPs found in whole milk powder and the protective effect of α-tocopherol in porcine ovarian granulosa cells.

CHAPTER 2

VALIDATION OF A GAS CHROMATOGRAPHIC METHOD FOR COPS DETERMINATION IN MILK POWDER

2.1 INTRODUCTION

COPs share very similar chemical structures, a property which requires that the analytical methods used for their detection possess a high resolving power. The presence of COPs at trace levels also demands that the methods be capable of sensitive detection (Park and Addis, 1992). Various methods have been developed for the analysis of COPs, from thin layer chromatography (TLC) to high-performance liquid chromatography (HPLC) or capillary gas chromatography (GC). A nuclear magnetic resonance technique (NMR) was also recently employed for this analysis in spray-dried egg powder (Fontana *et al.*, 1992, 1993). Trace analysis is complicated by interference from a diversity of other lipid components in food, in particular triglycerides, cholesterol and phospholipids (Schmarr *et al.*, 1996). Many of the problems that have been encountered in COPs analysis include loss of COPs during sample preparation (Csallany *et al.*, 1989), production of artefacts during handling (Rose-Sallin *et al.,* 1995), poor resolution or instability during sample preparation work-up and mis-identification (Table 2.1). In addition, quite considerable variation in fat content of powdered milk samples between different laboratories have been reported, suggesting a need to study different extraction methods (Appelqvist, 1996).

In view of the potential health impact of oxysterols in food, any discrepancies in results being reported, due to the use of different methods, may lead to consumer concern. In addition, the precise quantification of the oxysterols remains a challenging task, especially in foods containing low levels of these compounds (Rose-Sallin *et al.*, 1995). For COPs analysis, therefore, it is obvious that a dependable, reliable, robust and sensitive analytical method that will minimise the generation of artefacts is highly desirable. This chapter will provide an in depth review of the established methodology for COPs analysis and investigate the setting up and validation of a GC method for COPs detection. Table 2.2 lists various different methodologies that have been used in the analysis of COPs in

Table 2.1 Problems encountered in the chromatographic analysis of COPs.

Type	Example
Loss of COPs	cholesterol-α-epoxide during hot saponification
Production of artefacts	7-ketocholesterol to 3,5-cholestadiene-7-one.
Mis-identification	No use of Mass Spectrometry
Insolubility in non-polar solvent	Cholestanetriol is insoluble in petroleum ether
Poor resolution	Thin layer chromatography
Instability during lengthy procedures	Thin layer chromatography
Instability during gas chromatography	Production of dehydration products of COPs at high column temperatures
Poor detection by HPLC-UV detectors	cholesterol-α- and β- epoxides and their triol hydrolysis products

(Park and Addis, 1992)

Table 2.2 Qualitative and quantitative methods developed between 1985 and 1996 for analysis of COPs.

Reference	**Analytical Procedure**	**COPs Analysed**
Fisher *et al.* (1985)	Saponification/Al_2O_3/Si-acid/GC	Isomeric cholesterol-epoxides, epimeric 7-hydroxycholesterols & 25-hydroxycholesterol
Cleveland & Harris (1987)	Saponification/TLC	cholesterol-α-epoxide, 7-ketocholesterol, epimeric hydroxycholesterols and 25-hydroxycholesterol
Nourooz-Zadeh & Appelqvist (1988)	HIPextraction/Saponification/ Sep-Pak/TEAP Lipidex/GC-MS	Epimeric hydroxycholesterols and cholesterol-epoxides and 25-hydroxycholesterol
Sander *et al.* (1989)	Folch extraction/ Saponification/GC	Epimeric hydroxycholesterols and cholesterol-epoxides, 7-ketocholesterol, 25-hydroxycholesterol and cholestanetriol.
Chan *et al.* (1993)	Folch extraction/LC-Si Sep-Pak /GC	Epimeric hydroxycholesterols and cholesterol-epoxides and 7-ketocholesterol
Rose-Sallin *et al.* (1995)	Saponification/ Sep-Pak/ GC-MS	Epimeric hydroxycholesterols, 7-ketocholesterol, 25-hydroxycholesterol and cholestanetriol.
Nielsen *et al.*, (1996)	Folch extraction/ Sep-Pak/ HPLC/ GC-MS	Epimeric hydroxycholesterols and cholesterol-epoxides, 7-ketocholesterol, 25-hydroxycholesterol and cholestanetriol.

food during the past decade. All of the analytical methods used for COPs detection may be viewed as consisting of a series of stepwise procedures to accomplish the following goals (i) extraction of total lipids, (ii) enrichment of COPs, (iii) separation and detection of COPs and (iv) confirmation of structural identity (Park and Addis, 1992). Monitoring of artefacts, though also an important step within the analytical procedure is not always carried out, leading to possible overestimations of the risk of exposure to COPs.

(i) Extraction of total lipids

Milk contains 2 mg/g cholesterol (Nielsen *et al.*, 1995) representing approximately 0.3 % of total lipids, the remaining 99.70 % of milk lipids being mainly triglycerides. The preparation of sterol samples as cleanly as possible is obviously a crucial factor for the successful analysis of COPs in food and biological samples. The first step towards analysis can be achieved by extracting total lipids from samples with organic solvents in which cholesterol and its oxides are co-extracted. The strength of the extracting solvent is important to ensure a good extraction yield of COPs, whose polarities range widely from very non-polar to polar (Park and Addis, 1992). The most popular method over the past 4 decades has been the cold extraction procedure of Folch *et al.* (1957), in which samples are homogenized in a mixture of chloroform:methanol (2:1, v/v) as the extracting solvents. The ratio of sample to extracting solvent is usually 1:20 (w/v). The homogenates are filtered and the filtrates containing lipid extracts are washed with distilled water or salt solution to remove non-lipid material. The efficiency of various organic solvents to extract lipids and cholesterol has been studied (Kaneda *et al.*, 1980). Of the solvents tested, which included chloroform : methanol, benzene, acetone and diethyl ether, chloroform : methanol (2:1, v/v) was shown to be the most suitable solvent system to extract both lipids and cholesterol thoroughly. Other extraction systems have also been used: the modified Maxwell method (Maxwell *et al.*, 1987) is based on the principle that non-lipid components are absorbed on a mixture of celite and anhydrous sulphate while lipids that are not absorbed are eluted with a mixture of dichloromethane : methanol and determined gravimetrically. The modified Radin

method (Radin, 1981) employs a hexane : isopropanol (3:2 v/v) mixture to extract lipids for the analysis of COPs in butterfat, non-fat dry milk and heated butter.

(ii) Enrichment of COPs

The total lipid extract obtained from whole milk powder is a crude preparation, the major portion consisting of triglycerides and phospholipids while sterols are only a very minor component. Cholesterol oxides are present in milk powder at trace levels, if present at all (Nielsen *et al.*, 1995). The trace nature of COPs requires their enrichment from the bulk of the accompanying lipids in the total lipid extract. Following enrichment, COPs can be analysed with less interference and concentrated to above the detection limit. This enrichment is commonly carried out by either saponification or chromatographic means.

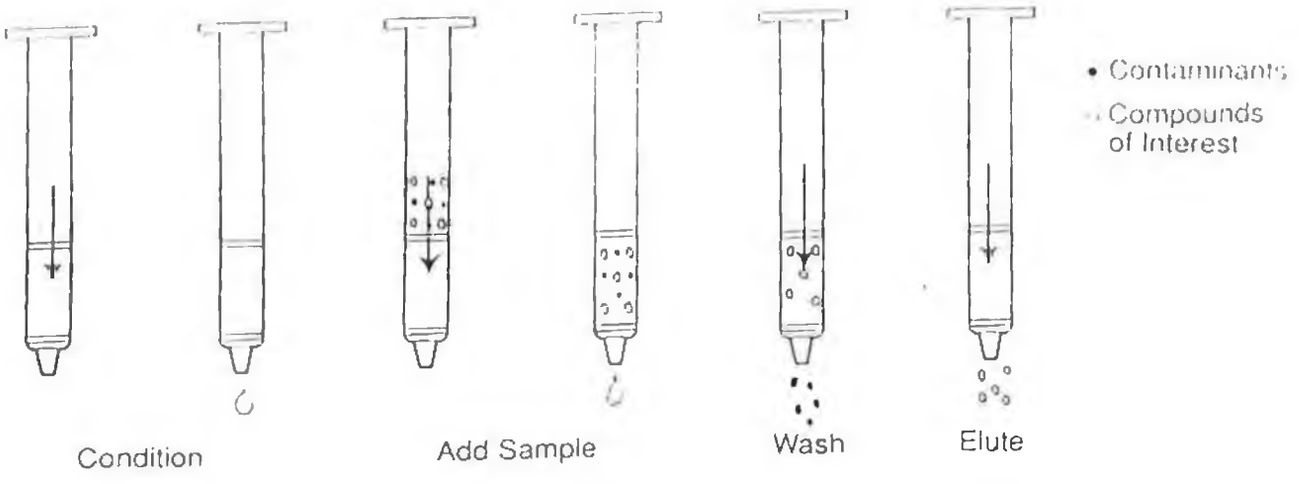
The basic principle of saponification (Fischer *et al.*, 1985; Cleveland and Harris, 1987) is to hydrolyse the ester bonds of trigylcerides and phospholipids in alkaline media such as methanolic or ethanolic KOH, converting them to water-soluble derivatives. To facilitate ester bond hydrolysis, the usual practice is to reflux the reaction mixture. After addition of water to the saponified mixture, COPs are extracted with suitable organic solvents (usually deperoxidised diethyl ether) into the non-saponifiable fraction. Variations in temperature and time of saponification exist; some workers saponify at 30° C for 2 h (Chicoye *et al.*, 1968), others saponify at room temperature for 2 h (Bascoul *et al.*, 1986) or overnight (Pie *et al.*, 1990). In addition, direct saponification of milk powders has been employed (Rose-Sallin *et al.*, 1995), although it is not very common. Nevertheless, a comparison of both direct saponification (where COPs are extracted from milk powders shaken for 18 h with 1M KOH in methanol) and indirect saponification (i.e. after lipid extraction) yielded no difference in the levels of COPs analysed (Appelqvist, 1996). Hot saponification is limiting because both 7-ketocholesterol and α-epoxide are unstable (Van de Bovenkamp *et al.*, 1988; Rose-Sallin *et al.*, 1995).

Column chromatography is another useful means of achieving enrichment of sterols from total lipid extracts (Schmarr *et al.*, 1996). Bond-Elut systems for extraction of

lipids may be based on diol, silica or aminopropyl sorbents. The interaction between lipid components and the adsorbent in the column for a given solvent or solvent system is the key factor determining the efficiency of isolating COPs or of eliminating other interfering substances. A schematic diagram of the solid-phase extraction apparatus used in enrichment of COPs is shown in Fig. 2.1.

A number of solid-phases can be used, though the use of aminopropyl has been recommended by a number of workers (Morgan and Armstrong, 1987; Chan *et al.*, 1993; Rose-Sallin *et al.*, 1995; Appelqvist, 1996). An aminopropyl sorbent takes advantage of the ionic character of fatty acids and phospholipids. The initial elution of neutral lipids leaves fatty acids and phospholipids on the NH_2 sorbent. The COPs are the least polar of the isolates and can be selectively eluted with acetone.

Although silicic acid column chromatography has been used for the separation of different lipid classes, only a few researchers have employed this method as a purification procedure in place of saponification prior to COPs analysis. Park and Addis (1985b) developed a procedure to analyze most of the more common COPs. After irrigating different lipid classes on silica gel plates with different developing solvents, they found that cholesterol and its oxides rarely migrated on silica gel plates using 10 % and 20 % ethyl acetate in hexane, whereas esterified cholesterol and triglycerides migrated rapidly. With acetone as the developing solvent, sterol fractions ranging from cholesterol to cholestanetriol migrated almost with the same mobilities but phospholipids remained at the origin due to their insolubility in acetone. Based on those observations, column chromatographic purification procedures were optimised (Smith, 1981): two consecutive washings with 28 ml hexane : ethyl acetate (9:1 v/v) and 20 ml hexane : ethyl acetate (8:2, v/v) through 4 g silica gel columns removed triglycerides and much of the cholesterol. Subsequent elution with 45 ml acetone released the COPs fraction free from phospholipid contamination. This purification procedure was later improved by De Vore (1988) who used commercially available disposable columns for the analysis of 7-ketocholesterol. With 2.8 ml volume silica columns, solvent consumption was



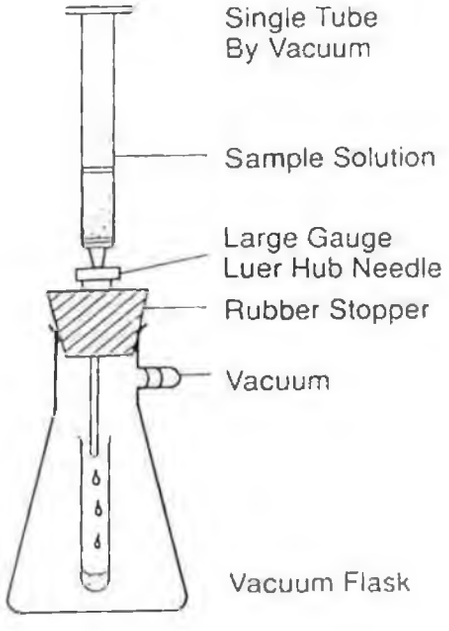


Fig. 2.1. Solid-phase extraction apparatus. The vacuum is used to pull solvent of interest through the Sep-Pak cartridge.

reduced to 2 ml for each washing and elution (De Vore, 1988).

(iii) Separation and detection of COPs

The chromatographic analysis of COPs began in the 1960s with thin layer chromatography (TLC) (Fioriti and Sims, 1967; Chicoye *et al.*, 1968) as the pioneering chromatographic method. Smith (1981) developed the method and reported Rf values for COPs after developing a mixture of oxides in heptane:ethyl acetate (1:1 v/v), spraying with 50 % H_2SO_4 and heating cautiously to 110-120° C. The brilliant colours that developed ranged from magenta for cholesterol through blues for diols and epoxides to yellow for triols. However, resolution with TLC can be poor, particularly for 7-ketocholesterol and the epimeric cholesterol α- and β-epoxides. In addition, it is not the method of choice today because it allows exposure of the samples to potential oxidation as a result of its large surface area and low loading capacity (Cleveland and Harris, 1987). The advent of High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) saw their subsequent use as analytical tools in COPs analysis.

Both reverse phase (Park and Addis, 1985b; Csallany *et al*, 1989) and normal phase (Maerker 1986; Tsai *et al*, 1980) HPLC systems have been used for COPs analysis. Good separations have been achieved with hexane : isopropanol combinations in which the hexane phase comprised 90-99 %. In spite of very significant advances during the past ten years, HPLC techniques have not reached the point where they are useful in the separation and quantification of complex mixtures of COPs (Maerker, 1986). Problems that have been encountered include excessive retention times for cholestanetriol (Maerker, 1986), poor sensitivity (Tsai *et al*, 1980) and inability to detect cholestanetriol by HPLC-UV detection.

Application of GC methods has been somewhat more successful despite problems of incomplete derivatisation and peak resolution. Both packed column GC (Tsai and Hudson, 1984) and capillary column GC (Park and Addis, 1985a ; Missler *et al*, 1985; Nourooz-Zadeh and Appelqvist, 1987) have been used in COPs detection. Capillary GC, especially in combination with direct on-column injection, has

increased the popularity of GC as a powerful tool (Maerker and Unrah, 1986). Nourooz-Zadeh and Appelqvist (1988) described a GC method for quantification of COPs in milk powders which was found suitable for quantifying the most abundant COPs in whole milk powders. In recent years, newer and more powerful chromatographic methods combined with MS (Nielsen *et al.*, 1995, 1996; Rose-Sallin *et al.*, 1995; Schmarr *et al.*, 1996) and NMR (Fontana *et al.*, 1992; 1993) have facilitated the definitive identification and quantification of cholesterol oxidation end products in foods and biological tissues.

GC accomplishes separation of components by partitioning the components of a chemical mixture between a mobile gas phase and a stationary phase held on a solid support (Willard *et al.,* 1988). Some sensitive compounds undergo partial thermal decomposition in the GC. Derivatisation may be employed to yield a more stable product and thereby improve chromatographic performance and peak shape (Kim and Nawar, 1991). Derivatisation also serves to accentuate the differences in the sample compounds to facilitate their chromatographic separation. The derivatisation most commonly used in the analysis of lipids is silylation. Silylation significantly increases volatility, especially in compounds with multiple polar groups. Silyl derivatives are formed by replacement of active hydrogen on OH, SH and NH groups (Willard *et al.*, 1988). Although silyl derivatives have the undesirable effect of fouling flame ionisation detectors (FID) with silica deposits, especially when samples are injected in excess reagent, these deposits may be reduced by use of fluorine containing reagents such as N,O-bis (trimethylsilyl) trifluoracetamide (BSTFA).

The instrument modules of a GC are shown systematically in Fig 2.2. A GC consists of several basic modules joined together to (i) provide a constant flow of carrier (mobile phase) gas, (ii) permit the introduction of sample vapours into the flowing gas stream, (iii) contain the appropriate length of stationary phase, (iv) maintain the column at the appropriate temperature (or temperature programmed sequence), (v) detect the sample components as they elute from the column, and (vi)

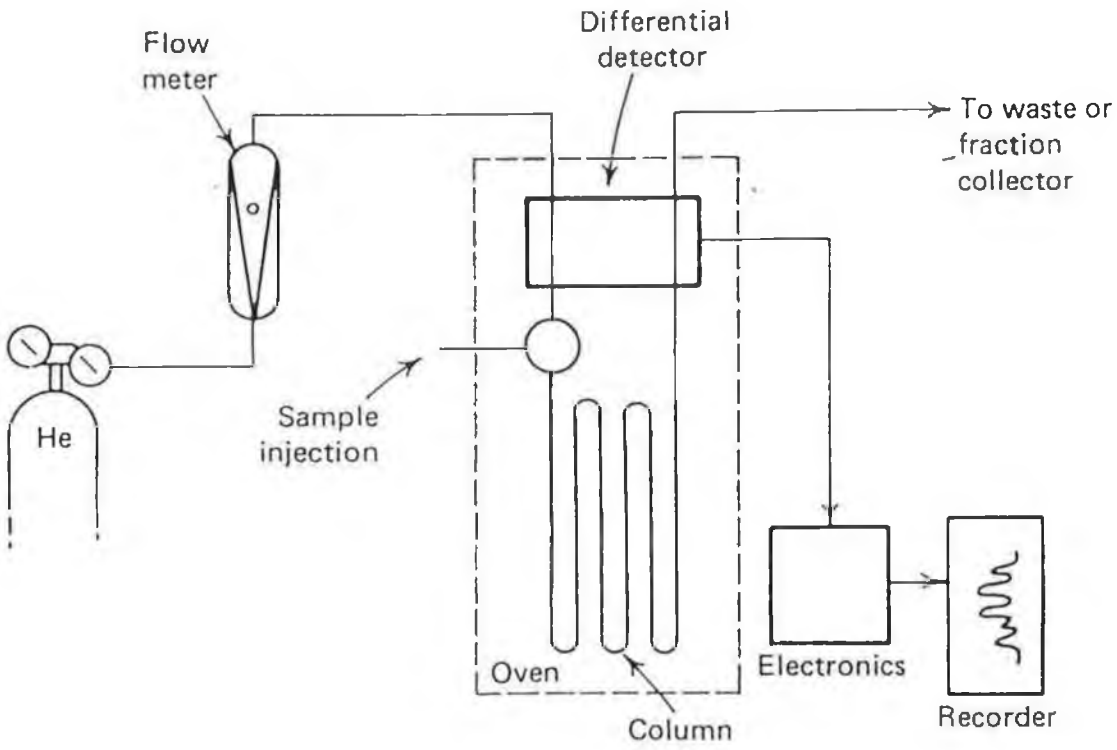


Fig. 2.2 Schematic of a Gas Chromatograph.

provide a readable signal proportional in magnitude to the amount of each component. The columns on which the components are separated are the key to good analysis, and are responsible for much of the versatility of gas chromatography. Temperature programming is another useful facility on many instruments. If the sample to be analysed contains components differing widely in volatility, it is advantageous to start the analysis with the column at low temperature so that the more volatile components are separated as single coherent peaks and then to raise the temperature at a fixed reproducible rate so that the less volatile components are eluted in a reasonable time. The sample emerges from the column into the detector, which is maintained, by a separate temperature control, at a slightly higher temperature than that reached by the column, so ensuring that there is no change in the response of the detector during temperature programming. FID is chosen most frequently for lipid analysis. Eluted components are burned in a flame of hydrogen and air and form ions that are detected and measured by an electrical system. Although the mechanism of the ionisation process is not fully understood, this detector is very sensitive, has good signal-to-noise ratio, is rugged and does not deteriorate significantly with prolonged use (Christie, 1982).

Quantitative GC demands that all compounds of interest in the samples injected produce reproducible peaks for integration. Accordingly, one needs to control possible sources of error such as sampling error, column performance and detector performance. Variability in the injection amounts can be compensated for by the use of the internal standard (IS) instead of the absolute analyte response. The IS should not be an original constituent of the analyte, should be separable from the analyte peaks and should have a chemical structure similar to that of the analytes. The use of such an IS will compensate for any losses incurred throughout the procedure, since the concentration ratio of the analytes to the IS can be reasonably assumed to remain constant (Willard *et al.*, 1988; Park and Addis, 1992). The most commonly used IS is 19-hydroxycholesterol (Sander *et al.*, 1989; Pie *et al.*, 1990; Rose-Sallin *et al.*, 1995; Appelqvist, 1996), though others such as 5α-cholestane (Nourooz-Zadeh and Appelqvist, 1988), 6-ketocholesterol (Chan *et al*, 1993; Lai *et al.*, 1995a) and

deuterium-labeled oxides (Rose-Sallin *et al.*, 1995) have also been used for the analysis of COPs in whole milk powder, egg powder, cake mixes and meat products.

(iv) Confirmation of structural identity

The presence of COPs can be confirmed using mass spectrometry (MS). Indeed, for quantification of low concentrations of oxysterols or for the analysis of samples with a high matrix background, verification of peak assignment by GC-MS is mandatory (Schmarr *et al.*, 1996). In GC analysis of COPs, identification of COPs is based upon comparing the retention time of a separated peak with that of an authentic standard. Because of the possibility that more than one compound can elute with a retention time the same or very close to that of the target compound, identity confirmation of a chromatographically separated peak needs to be performed in a more stringent manner, i.e. by means other than retention time comparisons. Nielsen *et al.* (1995) reported that oxidised monoacylglycerols and other compounds coeluting with the oxysterols can result in an overestimation of the level of oxysterols when analysed using GC-FID, but not when using the MS selected ion monitoring technique. MS is commonly used and provides far more precise information on chemical identity of COPs (Rose-Sallin *et al.*, 1995; Nielsen *et al.*, 1995).

In MS analysis, analytes are introduced into the ionization chamber either via the chromatographic inlet or by a direct insertion probe. The stream of molecules introduced in an ionization chamber is bombarded by high-energy electrons emitted from a heated filament, with a voltage of approximately 70 eV. As a result, very small amounts of molecules are ionized and the ionized molecules may remain as such or undergo characteristic fragmentation producing a variety of ions. These ion beams are separated through a mass analyser according to their mass/charge (m/z) ratio. The molecular ion (m^+) is a radical cation formed from the ionization of an analyte molecule with the loss of one electron. The molecular ion therefore, appears at an m/z value numerically equal to the nominal molecular weight of the compound. The most intense peak in the mass spectrum is called the base peak, against which the relative intensities of all other ions are normalized (Park and Addis, 1992).

(v) Monitoring of artefact formation

The critical step in the analysis of COPs is the clean-up procedure which should be designed to minimise the generation of artefacts (Rose-Sallin *et al.*, 1995). The formation of COPs by autoxidation of cholesterol and their subsequent instability require a very mild analytical method to prevent artefact formation. The presence of air, light, peroxides in solvents or heat treatment during the clean-up procedure can promote artefact formation. The activity of some adsorbing agents such as silica gel is another possible reason for artefact formation, due to the interaction of cholesterol with silicic acid in the presence of air. Moreover, the pH of the media can influence the stability of several COPs (Kim and Nawar, 1993) in particular 7-ketocholesterol, which is very sensitive to hydrolysis in hot alkaline media (Chicoye *et al.*, 1968). Deuterium-labelled cholesterol has been used for spiking samples prior to analysis (Rose-Sallin *et al.*, 1995). The extent of oxidation occurring during the clean-up procedures can be easily determined from the deuterated COPs formed. The difference between unlabelled COPs and deuterated COPs allowed determination of artefact-free COPs content of a sample. Artefact formation during milk powder analysis has been reported to be approximately 2 % (Rose-Sallin *et al.*, 1995), which underlines the importance of a mild analytical procedure for COPs analysis.

2.2 AIMS

To set up and optimise a GC method for COPs determination in milk powders

2.3 OBJECTIVES

- Evaluation of the performance characteristics of a capillary GC column for separation and resolution of a number of COPs.

- Confirmation of the identity of COPs using GC. MS.

- Use of the established method for screening a variety of milk powders produced under low-, medium- and high-heat treatments.

2.4 EXPERIMENTAL

2.4.1 REAGENTS

Cholest-5-ene-3β-ol (cholesterol), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), cholest-5-ene-3β,25-diol (25-hydroxycholesterol), cholesterol-5α,6α-epoxide (cholesterol-α-epoxide), cholest-5-en-3β,19-diol (19-hydroxycholesterol), cholestane-3β, 5α, 6β-triol (cholestanetriol), all of > 99 % purity, N,O-bis (trimethylsilyl) trifluoracetamide (BSTFA) and cysteine were purchased from Sigma (Poole, Dorset, U.K.) and cholesterol 5β,6β-epoxide (cholesterol-β-epoxide) was purchased from Steraloids Inc. (Wilton, NH, USA). Malondialdehyde bis diethylacetal (MDA) was purchased from Merck-Schwchardt, Darmstadt, Repubic of Germany. The cholesterol kit (Cat. No. 139 050) was purchased from Boehringer Mannheim (Mannheim, Germany). Ellman's reagent (5, 5 dithiobis-(2-nitrobenzoic acid)) was purchased from Aldrich-Chemie (Steinheim, Germany).

2.4.2 Standard Preparation

2.4.2.1 Preparation of standard solution of cholesterol and cholesterol oxides

A standard solution (standard mix I) containing approximately 10 mg of cholesterol, cholesterol-β-epoxide, cholesterol-α-epoxide, 7-ketocholesterol and cholestanetriol and 5 mg 25-hydroxycholesterol was prepared in a 20ml volumetric flask . The multistandard solution was made up to the 20 ml volume mark with chloroform. The final concentration of each oxide, except 25-hydroxycholesterol, was 500 μg/g. The concentration of 25-hydroxycholesterol in the multistandard solution was 250 μg/g. This standard mix was used to spike whole milk powder samples to monitor % recovery.

A second standard mix (standard mix II) was prepared for GC.MS analysis. A 1000 μg/g standard mix was prepared by adding exactly 10 mg of cholesterol, cholesterol-α-epoxide, 7-ketocholesterol, 25-hydroxycholesterol and cholestanetriol into a 10 ml volumetric flask and making up to the volume with chloroform. A 100 μg/g and 10

μg/g solution was then made by serial dilution of the 1000 μg/g standard mix. These were then used to spike whole milk powder samples to monitor the repeatability and reproducibility of the method.

2.4.2.2 Preparation of internal standard solution

Into a 20 ml volumetric flask 10 mg 19-hydroxycholesterol was weighed and made up to the volume with chloroform.

2.4.3 Determination of cholesterol in whole milk powder

The Boehringer Mannheim cholesterol assay kit (Cat. No. 139 050) was used to measure total cholesterol in milk powders. Approximately 10 g whole milk powder was weighed into a 250 ml round-bottomed flask and 50 ml freshly prepared methanolic 2 M KOH solution was added. The solution was heated for 30 min under reflux. The warm solution was transferred with 100 ml distilled water into a 1000 ml separatory funnel. After cooling to room temperature, the solution was shaken with 100 ml ether/petroleum ether. The clearly separated bottom phase was carefully drained off into a saponification flask and the organic phase transferred into a 500 ml round bottomed flask. This extraction was repeated twice. The combined ether/petroleum ether phases were evaporated under a rotatory evaporator at 35° C and the residue was dissolved in isopropanol and transferred into a 50 ml volumetric flask, filtered and the clear solution then used for the cholesterol determination. The assay was performed by adding 2.5 ml of the sample solution to 0.02 ml of cholesterol reagent solution (95 ml ammonium acetate, 2.6 M methanol, 222,000 U catalase, 0.05M acetylacetone, 0.03M methanol) and incubating at 37° C for 60 min. The solution was then cooled at room temperature and the absorbance read at 405 nm. The fat was determined using the Rose-Gottlieb procedure and results were expressed in mg cholesterol/g fat.

2.4.4 Folch Extraction and derivatisation of COPs

For COPs analysis, 0.05 mg 19-hydroxycholesterol was used as an internal standard and was added to 1 g of milk powder to monitor recoveries and correct for losses during sample preparation. The method of Folch *et al.*(1957), as modified by Sander *et al.* (1989) was employed for extraction of lipids for COPs analysis. 1 g milk

powder was added to 10 ml chloroform : methanol (2:1 v/v) and homogenised with a Polytron homogeniser for 2 min. A further 15 ml chloroform : methanol (2:1 v/v) was added and the mixture was homogenised again for 2 min. The homogenate was filtered through a Whatman no. 42 filter paper into a 50 ml centrifuge tube and 5 ml distilled water then added. The tube was centrifuged at 1000 g for 20 min, and the organic phase (lower) transferred into a 100 ml round bottomed flask. The organic phase was evaporated to dryness in a rotary evaporator at 30° C and dissolved in 5 ml hexane. This lipid extract was then applied to a Sep-Pak silica cartridge (Waters Associates, Milford, MA, USA) which had been previously equilibrated with 5 ml hexane. The solid phase extraction column was then washed successively with 10 ml hexane/diethyl ether (95:5 v/v), 25 ml hexane/diethyl ether (90:10 v/v) and 15 ml hexane/diethyl ether (80:20 v/v). The COPs fraction was eluted with 10 ml acetone and dried under nitrogen as described by Morgan and Armstrong (1989). The COPs were converted to trimethylsilyl ethers by addition of 250 µl BSTFA and silylation was completed by heating at 120° C for 30 min.

2.4.5 Measurement of COPs by Gas Chromatography

Separation of COPS was performed using a Varian 3500 gas liquid chromatograph (Varian, CA, USA) fitted with a flame ionization detector (FID) as described by Pie *et al.* (1990). Separation was performed on a DB-5 capillary column (J & W Scientific, Folson, CA) (30 m x 0.32 mm i.d., 0.25 mm film thickness). Samples were injected onto the column using an on-column injector (SPI), using He as carrier gas and N_2 as make-up gas and a flow-rate of 5 ml/min. Injector temperature was 100° C increasing to a final temperature of 300° C at a rate of 30° C/min. Initial column temperature was 100° C with a hold time of 40 min, after which it increased to 300° C at a rate of 10° C/min. The detector was at 300° C. Total COPs were detected in whole milk powder and then expressed as a fraction of the lipid content, which was determined using the Rose-Gottlieb procedure. COPs were expressed in µg/g fat.

2.4.6 Confirmation of identity by Gas Chromatography / Mass Spectrometry

The structures of COPs were confirmed by GC/MS, using a Varian Saturn 4D GC/MS (Varian, Harbor City, CA, USA). The conditions used were as previously outlined (Rose-Sallin *et al.*, 1995). Separation was performed on a DB-5 fused silica capillary column (J & W Scientific, Folson, CA, USA) (30 m x 0.32 mm i.d., 0.25 mm film thickness), following on-column injection, using He as carrier gas at 0.7 bar. The initial oven temperature was 60° C with a hold time of 1 min. This was increased at a rate of 30° C/min to 200° C which was then increased to 300° C at 5° C/min and held for 5 min. The mass spectrometer was operated under electron ionisation conditions with an electron energy of 70 eV.

2.4.7 Optimisation of the analytical procedure

The optimisation of the method required the following experiments:

(i) Determination of the optimum volume of BSTFA and derivatisation time for silylation of COPs by BSTFA.

(ii) Determination of the theoretical 100 % recovery of internal standard by direct injection of derivatised 50 μg 19-hydroxycholesterol.

(iii) Optimisation of the extraction of lipid extract for sample clean-up.

(iii) Establishment of the optimum procedure for Sep-Pak clean-up. A vacuum apparatus was set up with a controlled flow-rate and this was compared with uncontrolled vacuum elution of solvent.

2.4.8 Validation of GC method for COPs analysis

2.4.8.1 Linearity

Calibration curves were constructed for cholesterol, cholesterol-α-epoxide and cholesterol-β-epoxide, 7-ketocholesterol, 25-hydroxycholesterol and cholestanetriol in the range 0-600 μg/g on a DB-5 column using a Varian 3500 GC.FID (National Dairy Products Centre, Moorepark, Fermoy, Co. Cork). Calibration curves were also constructed in the range 0-1000 μg/g using the Varian Saturn 4D GC.MS (Tralee Regional Technical College, Tralee, Co. Kerry).

2.4.8.2 Response factors

The response factors were calculated relative to the IS. Relative response factors were calculated as described by Van de Bovenkamp *et al.* (1988) as follows:

Response Factor = $(W_n / A_n)/(W_{is}/A_{is})$

W_n and W_{is} are weights of the nth COP and IS, respectively

A_n and A_{is} are peak areas of nth COP and IS, respectively

Peak height can also be used in lieu of peak area. After the GC run of samples, the concentration of each oxide (μg/g) was calculated using the following equation:

Concentration = (A_i / A_{is}) RF (W_{is} / W_i)

A_i and W_i refer to the peak area and weight of sample.

2.4.8.3 Recovery

To evaluate the recoveries of the different oxides, 1 g of fresh whole milk powder (containing no COPs) was spiked with 500 μl of standard mix solution (standard mix I) containing cholesterol and COPs. The samples were taken through the entire procedure and analysed by GC. Recoveries were calculated by expressing recovered amounts of individual oxides as a percentage of the original spiked amounts (standard mix I).

2.4.8.4 Precision

Precision of the method was established by determining the repeatability and reproducibility of the method. Fresh whole milk powder (containing undetectable levels of COPs) was spiked with 500 μl of 10, 100 and 1000 μg/g standard mixture of COPs in triplicate on the same day (repeatability) and on three consecutive days (reproducibility). The coefficient of variation (% CV) was calculated to measure the degree of precision obtained.

2.4.8.5 Limit of Detection

The limit of detection (LOD) was determined by firstly establishing the base noise level by running a number of solvent blanks. The LOD was determined as the concentration whose signal height is three time that of the noise.

2.4.9 Powder Sampling

Milk powder samples were received from a number of Co-operatives in Ireland. The milk powders were divided into varying heat classes. Low-heat powders received a heat treatment of less than 75°C for 10 sec; medium-heat powders received a heat treatment of between 76.5-85°C for 15-30 min and high-heat powders received a heat treatment of greater than 110 °C for 2 min. All samples were analysed on receipt with fresh milk powders being analysed within a week of manufacture. Stored milkpowders refer to powders stored for longer than 12 months. Different batches were analysed for each category and the results pooled for statistical analysis.

2.4.10 Statistical Methods

The Mann-Whitney test was used to determine the statistical significance of the differences in COPs levels between low-heat, medium-heat and high-heat powders, using the GENSTAT package (The Numerical Algorithims Group Ltd., Oxford, UK).

2.5 RESULTS

Prior to commencing the study to determine total and individual levels of COPs in milk powders, it was necessary to characterise all stages of the method. Thus, optimisation of the extraction and clean-up stages before GC analysis; precision of GC separation method; confirmation of the identity of COPs; evaluation of the linearity and recovery of each oxide were evaluated in order to validate a method to measure COPs in milk powders. The accuracy of the entire procedure was assessed by spiking milk powders with varying amounts of each oxide and assessing recoveries, reproducibility and repeatability of the method.

2.5.1. Retention times and relative response factors

Using the parameters described in section 2.4.5, the complete separation of COPs was achieved by non-polar capillary column (DB-5) (Fig. 2.3). Retention times and response factors relative to 19-hydroxycholesterol are shown in Table 2.3. Response factors (RF) ranged from 0.70 ± 0.02 to 1.38 ± 0.03. Cholesterol had the shortest retention time (26.7 min) and cholestanetriol the longest (37.1 min). The COPs eluted in the following order: 19-hydroxycholesterol, cholesterol-β-epoxide, cholesterol-α-epoxide, 25-hydroxycholesterol, 7-ketocholesterol and cholestanetriol.

Mass spectrometric detection of the compounds was achieved after electron impact (EI) ionisation. Fig 2.4 to 2.6 are mass spectra for TMS ethers of 7-ketocholesterol 25-hydroxycholesterol cholesterol, 19-hydroxycholesterol, cholestanetriol, cholesterol-α-epoxide, and. The molecular ions for each of the oxides are also outlined in Table 2.3. Mass spectrum analysis of 7-ketocholesterol resulted in ions at m/z 174 (base peak) and at m/z 159 and 456 while that of 25-hydroxycholesterol resulted in a base peak at m/z 131. The m/z ions of interest were m/z 143 and 445 in the mass spectrum of cholestanetriol, the only COPs among those studied to have three hydroxyl groups. The mass spectrum of cholesterol was dominated by the ion of m/z 129 which is the base peak. The m/z 129 originates from the A-ring fragmentation between C1-10 and C3-4 (Park and Addis, 1992). Other distinguished fragment ions were at m/z 253 and 351.

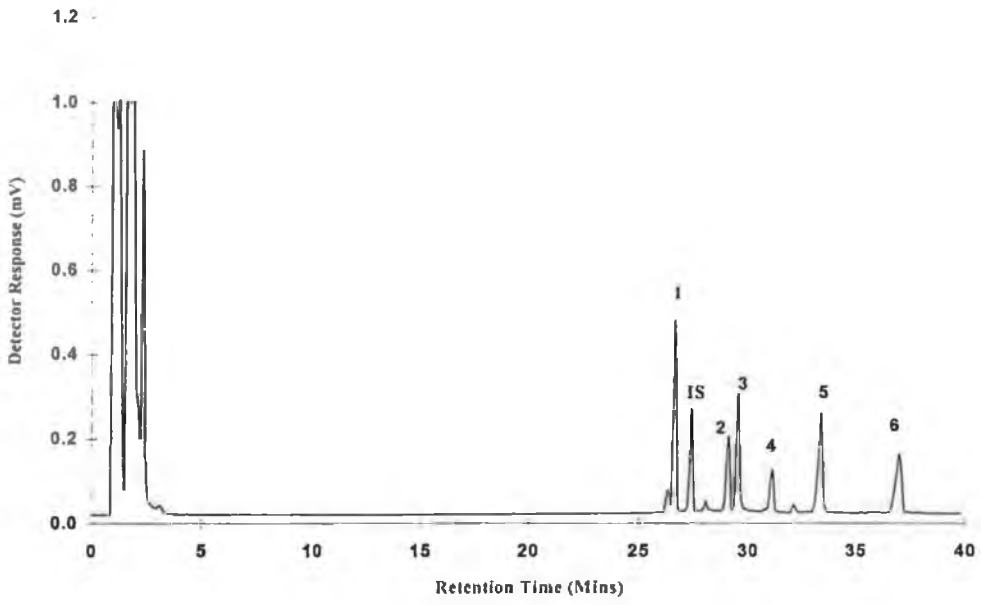


Fig. 2.3. Chromatogram of COPs standard mix, with 19 hydroxycholesterol as the internal standard (IS). (1) Cholesterol; (2) cholesterol-β-epoxide; (3) cholesterol-α-epoxide; (4) 25-hydroxycholesterol; (5) 7-ketocholesterol and (6) cholestanetriol.

Table 2.3. Retention times, relative response factors and molecular ions for cholesterol and COPs of interest.

COPs	Retention Time (min)	Relative Response Factor [a]	Mass Spectrum Ions (m/z)
Cholesterol	26.70	0.79 ± 0.012	129; 253; 351
19-hydroxycholesterol	27.33	1.00 ± 0.022	145; 197; 353
cholesterol-β-epoxide	28.71	1.21 ± 0.018	NA
cholesterol-α-epoxide	29.60	0.98 ± 0.008	135; 366
25-hydroxycholesterol	31.11	0.70 ± 0.016	131
7-ketocholesterol	33.39	1.02 ± 0.026	174; 159; 456
Cholestanetriol	37.08	1.38 ± 0.032	143; 445

[a] n (number of analysis)=6

NA = Not Available

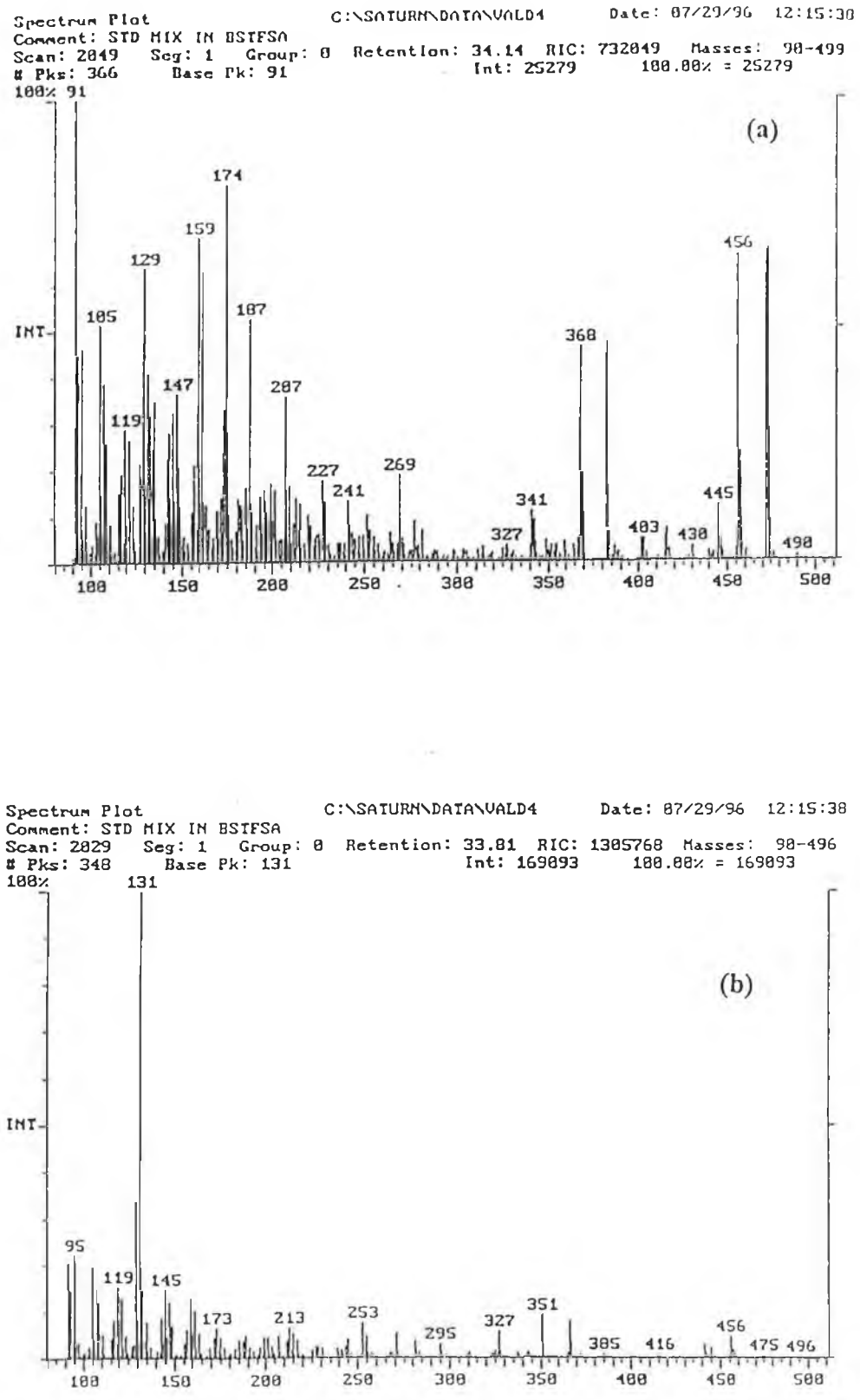


Fig. 2.4 Mass spectra of (a) 7-ketocholesterol and (b) 25-hydroxycholesterol TMS ethers.

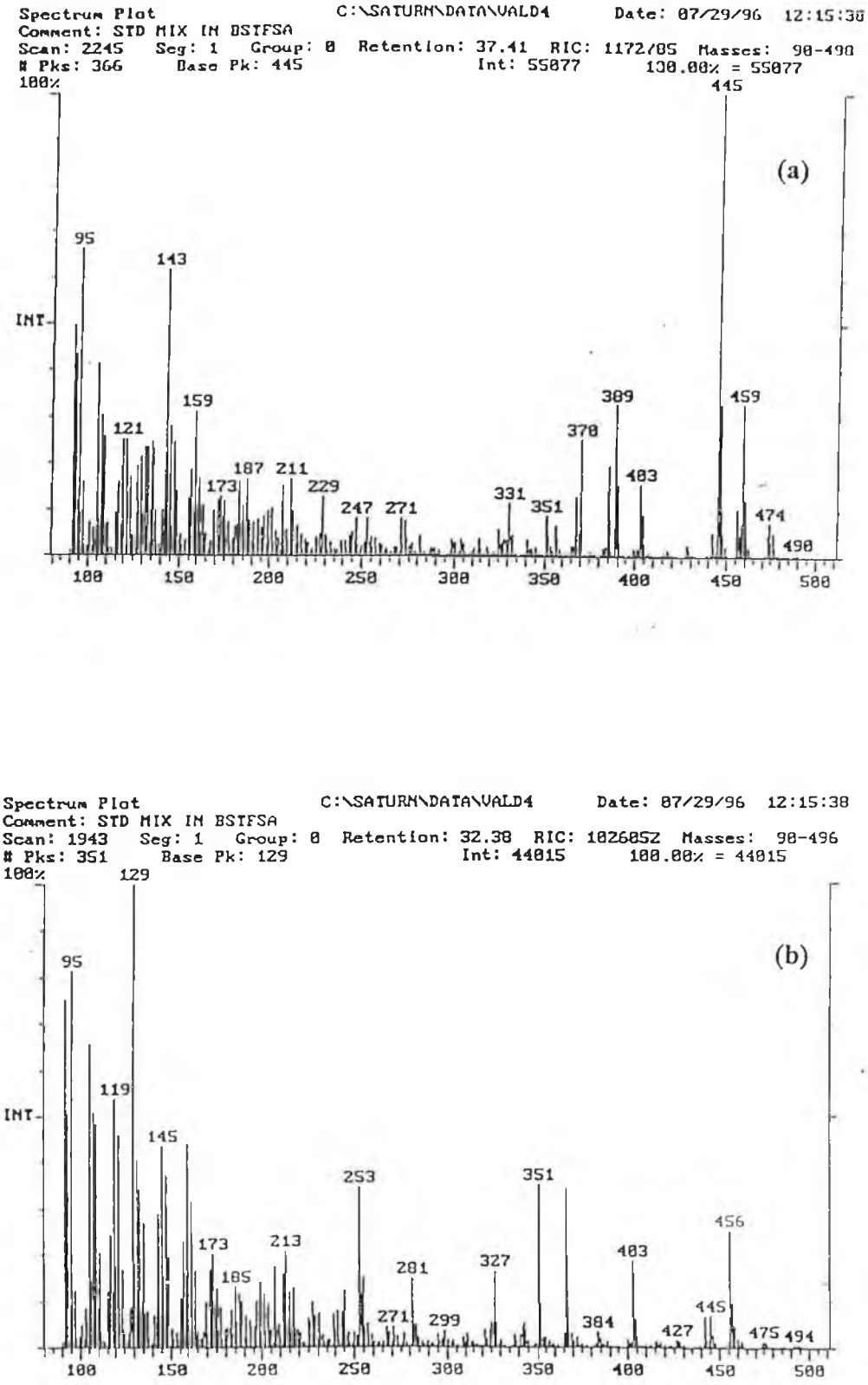


Fig. 2.5 Mass spectra of (a) cholestanetriol and (b) cholesterol TMS ethers.

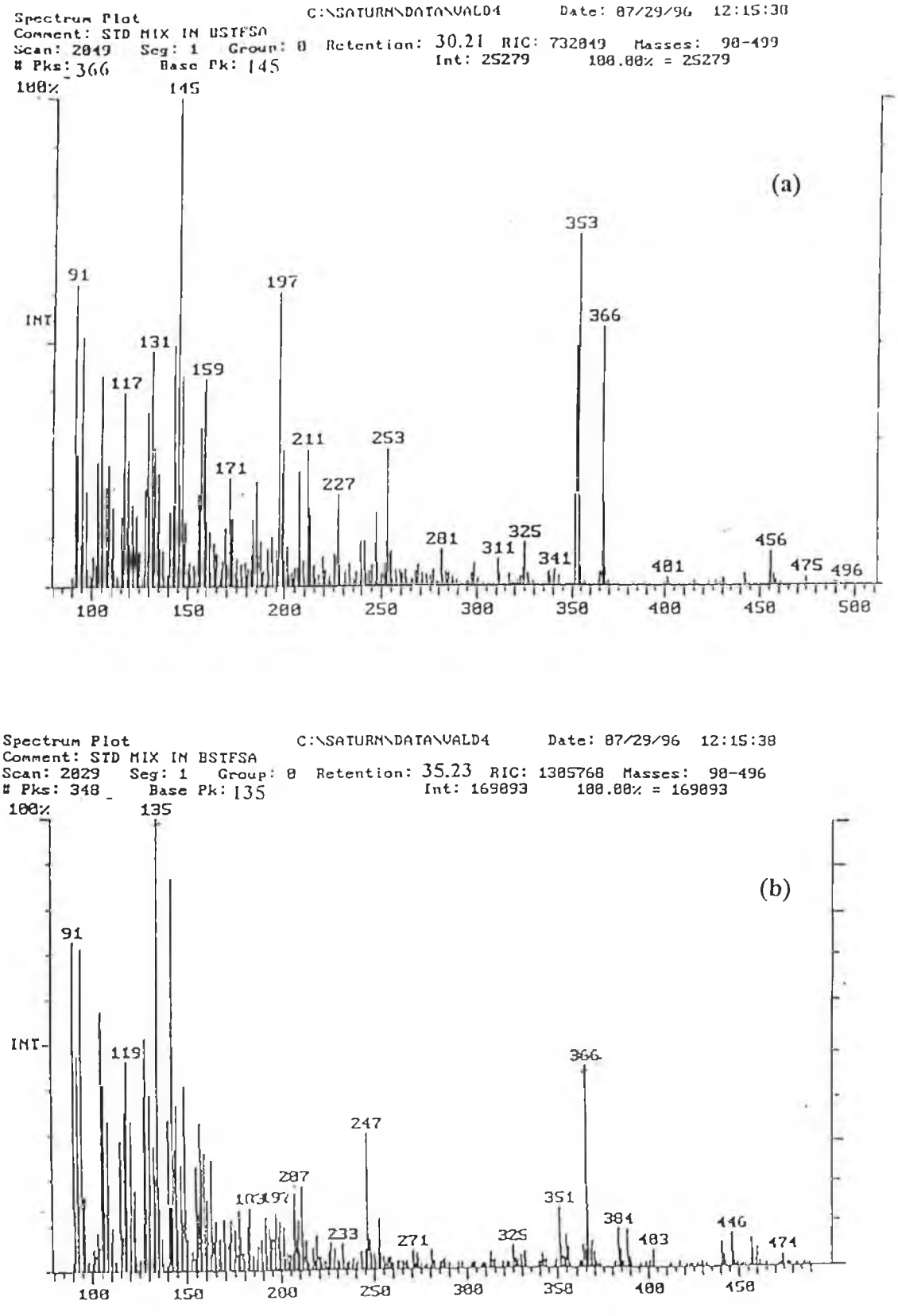


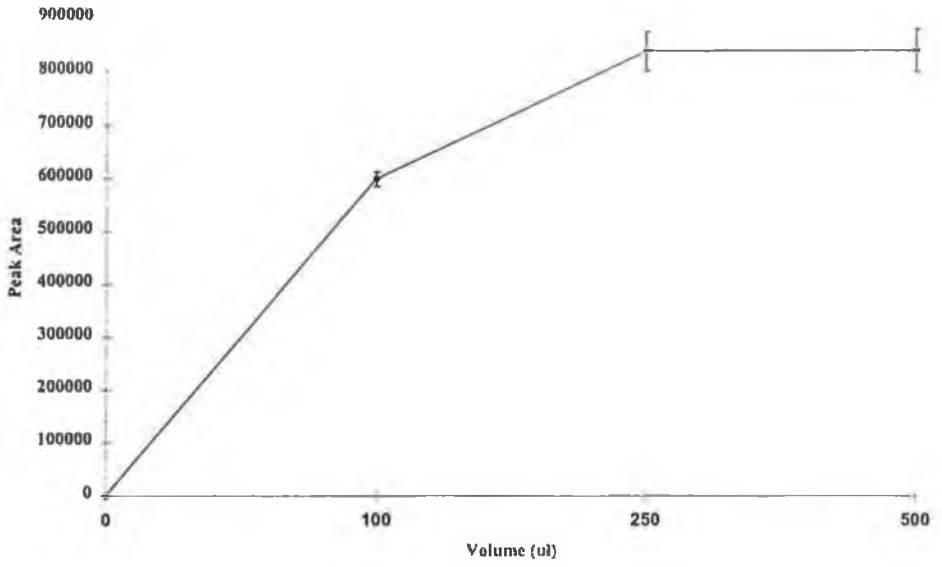
Fig. 2.6 Mass spectra of (a) 19-hydroxycholesterol and (b) cholesterol-α-epoxide TMS ethers.

The mass spectrum of 19-hydroxycholesterol was dominated by ions at m/z at 145, 197 and 353. Mass spectrometric analysis of α-epoxide resulted in extensive fragmentation, leaving a limited number of ions in the high m/z region with the major ions at m/z 135 and 366.

2.5.2 Recovery of 19-hydroxycholesterol after extraction and enrichment

The GC parameters employed in this work for analysis of COPs in whole milk powder are described in Table 2.3. The entire analytical procedure for COPs quantification from extraction to derivatisation was optimised before milk powders were analysed for their COPs content. 19-hydroxycholesterol was used to monitor the procedure. The theoretical 100 % recovery was first established by injecting derivatised 19-hydroxycholesterol directly onto GC and recording the peak area. Its recovery was then assessed after (i) extraction, (ii) clean-up on a Sep-Pak cartridge and (iii) derivatisation. BSTFA was used under different volume-time conditions; the optimal volume of BSTFA was observed to be 250 µl and optimal length of incubation time required for derivatising 19-hydroxycholesterol (0.05 mg) into its trimethylsilyl (TMS) derivative was 30 min at 120° C (Fig 2.7). 50 µg 19-hydroxycholesterol (corresponding to 562310 peak area units) was added to milk powder and the lipid extracted with 25 ml chloroform : methanol (2 : 1 v/v) and dissolved in a single 5 ml aliquot of hexane. After drying down under N_2 and derivatisating with 250µl BSTFA for 30 min, 456,813 peak units was obtained, resulting in approximate 81 % recovery (Table 2.4). When a second wash with 5 ml hexane was included, the recovery improved from 81 % to 93 %. Approximately 60 % of added internal standard was recovered after application of the lipid extract to the Sep-Pak cartridge with its elution under uncontrolled conditions (application of high pressure to accelerate flow-rate). However, using a controlled flow-rate and double hexane wash, recovery of internal standard was approximately 77 %. Though an approximate 23 % loss of 19-hydroxycholesterol occurred after the clean up stage, it was nevertheless considered necessary to continue with the solid-phase extraction as it considerably cleaned up the lipid extract prior to its injection on the GC column. Fig 2.8 shows a GC chromatogram of a whole milk powder sample before and after clean up.

(a)



(b)

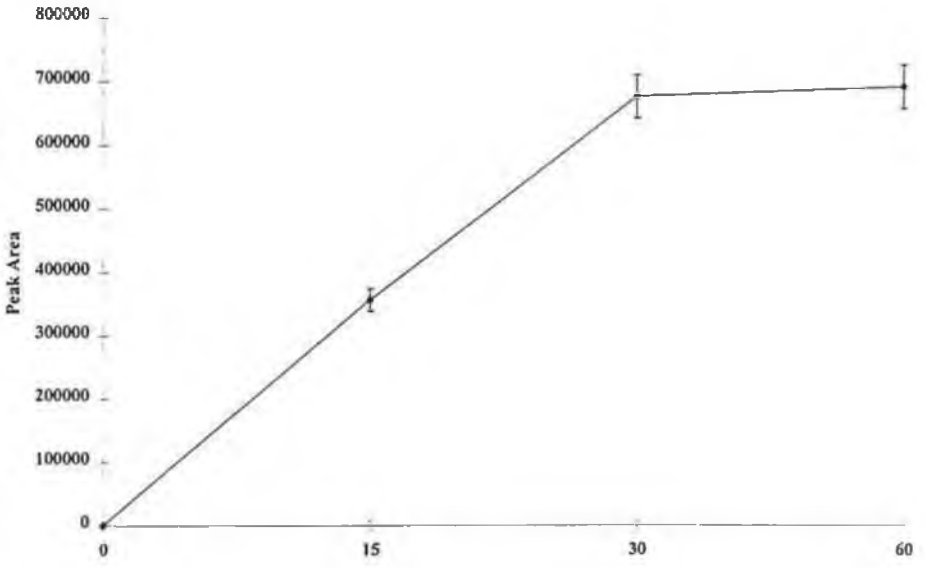


Fig 2.7(a) The optimal volume of BSTFA required for derivatisation of 19-hydroxycholesterol and (b) time course assay for the derivatisation of 19-hydroxycholesterol to its TMS ether. Error bar refer to mean values ± standard deviation (n=6).

Table 2.4. % Recovery of 19-hydroxycholesterol (a) before and (b) after optimisation.

(a)

Sample	Peak Area [a]	Recovery %
50 μg IS [a]	562, 310 ± 5699	100
Extraction and single hexane wash Sep-Pak (manual uncontrolled flow-rate)	456,813 ± 6137	81.2
	337, 150 ± 5129	59.9

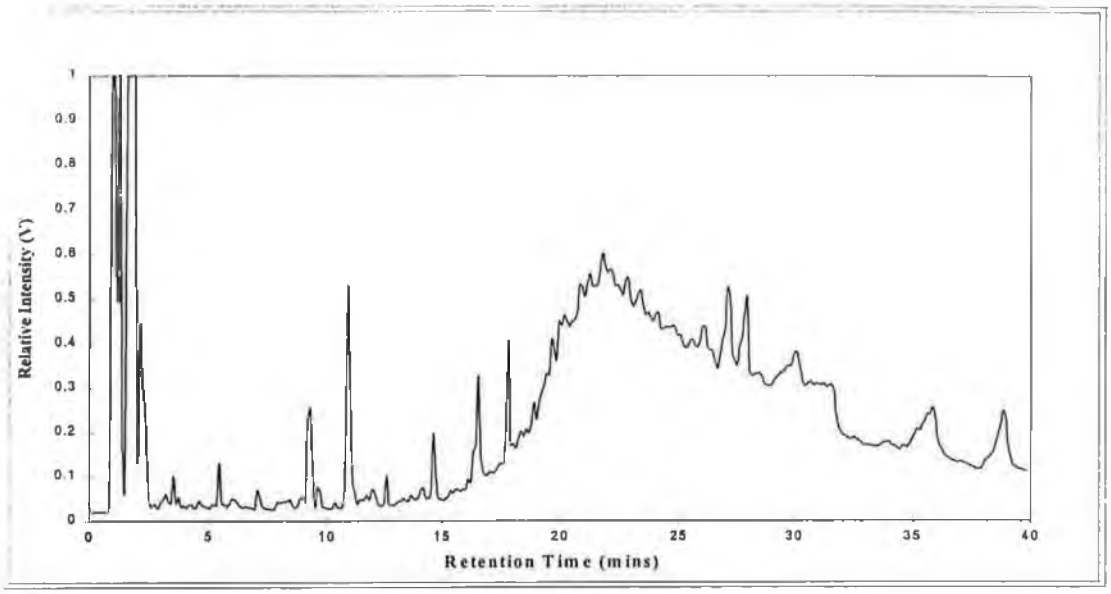
(b)

Sample	Peak Area	Recovery %
50 μg IS [a]	562, 310 ± 5699	100
Extraction and double hexane wash Sep-Pak (controlled flow-rate at 5 psi)	520, 318 ± 4933	92.5
	431, 036 ± 8973	76.7

[a] n (number of analysis)=3

Data refer to mean ± standard deviation

(a)



(b)

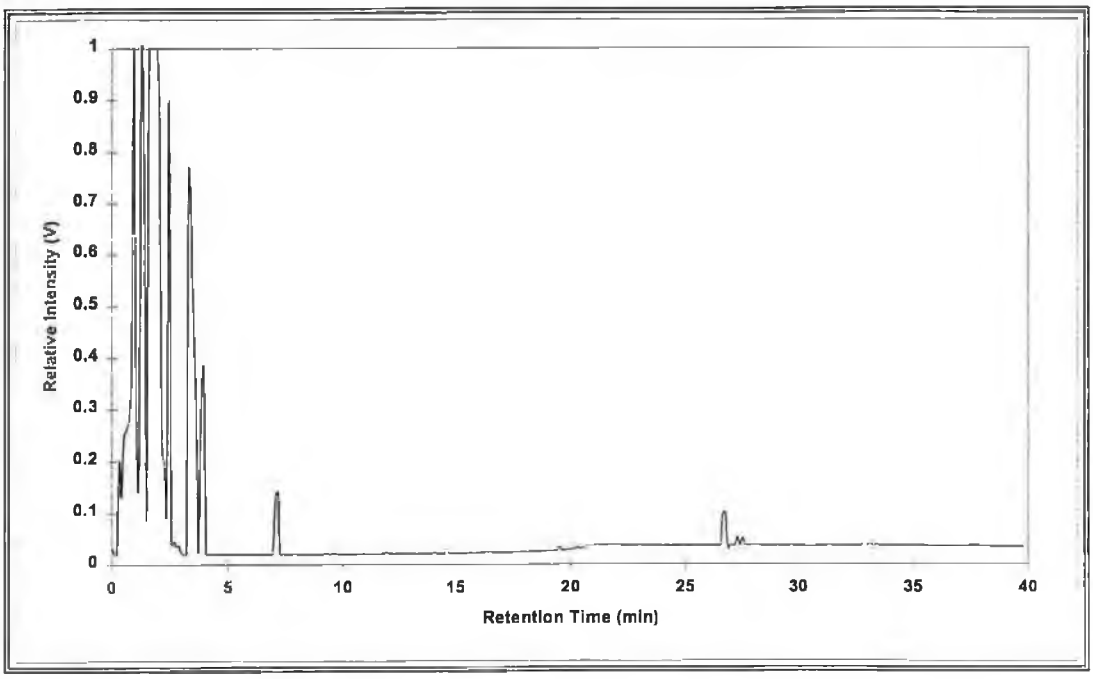


Fig. 2.8. Gas chromatogram of fresh (a) a low-heat whole milk powder sample without Sep-Pak clean-up and (b) having undergone a Sep-Pak clean up prior to injection

2.5.3. Linearity of response

Standard curves of COPs were constructed relating peak area to concentration (μg/g) of each oxide. Figs. 2.9-2.12 show the standard curves for cholesterol, 7-ketocholesterol, cholesterol-α-epoxide, 25-hydroxycholesterol, cholestanetriol and cholesterol-β-epoxide in the range 0 to 600 μg/g. Varying peak areas were obtained for each oxide and relative response factors were calculated as 'correction factors' to be used in their subsequent quantification in milk powders. Correlation coefficients of greater than 0.98 were obtained. The limit of detection was 0.1 μg/g lipid and was determined as three times the standard deviation of the noise.

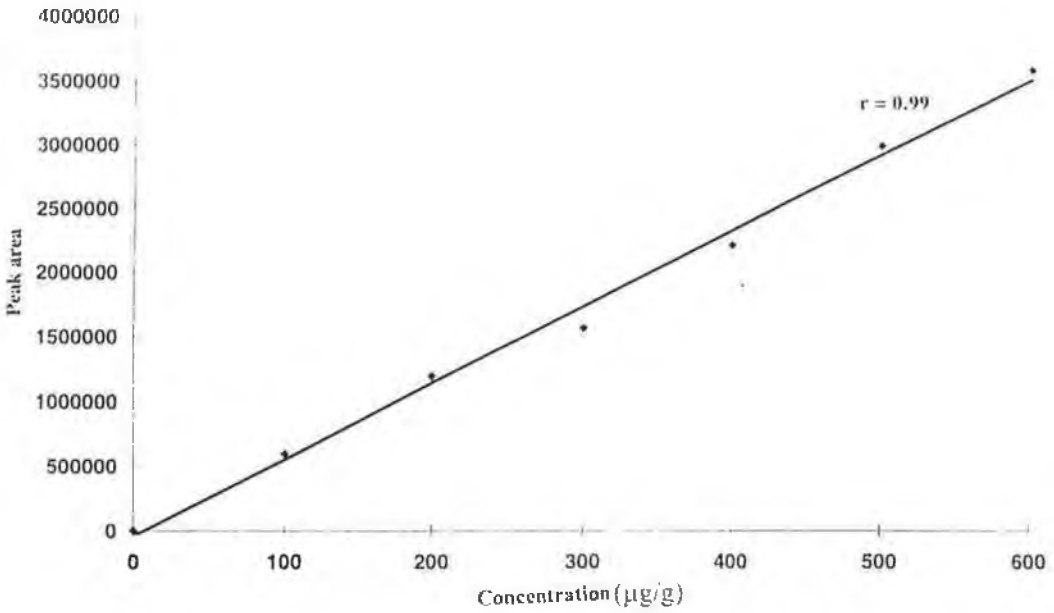
2.5.4 Recovery

Table 2.5 demonstrates the % recovery of cholesterol, cholesterol-α-and β-epoxides, 25-hydroxycholesterol, 7-ketocholesterol and cholestanetriol following extraction from whole milk powder and subsequent analysis by GC. Fresh low-heat milk powder, containing no detectable COPs was spiked with a standard mix containing 0.05 % w/v COPs and 0.025 % w/v 25-hydroxycholesterol and analysed for COPs. The resultant peak areas were compared with peak areas of the pure standard mix analysed directly by GC. Recovery of each COPs with the exception of cholestanetriol (59 %) was greater than 90 % and there were negligible differences in the amounts of each oxide recovered.

2.5.5 Precision

Precision of the method was determined by evaluating the intra-and inter-assay variations, respectively. Fresh low-heat whole milk powder was spiked with 500 μl of 10, 100 and 1000 μg/g of a standard mix solution (standard mix II) of COPs in triplicate on the same day (intra assay) to determine the repeatability of the method. The coefficients of variation (% CV) (Table 2.6-2.10) were shown to range from 3.23 to 5.29 % for powders spiked with 10 μg/g of each oxide.

(a)



(b)

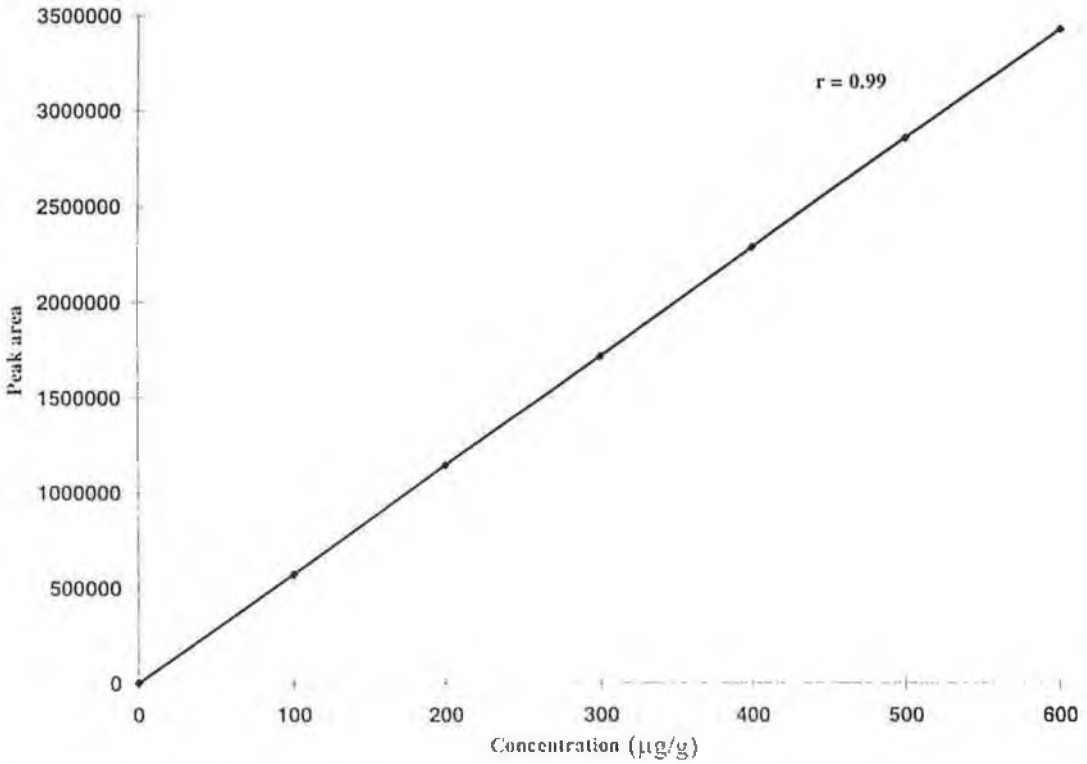
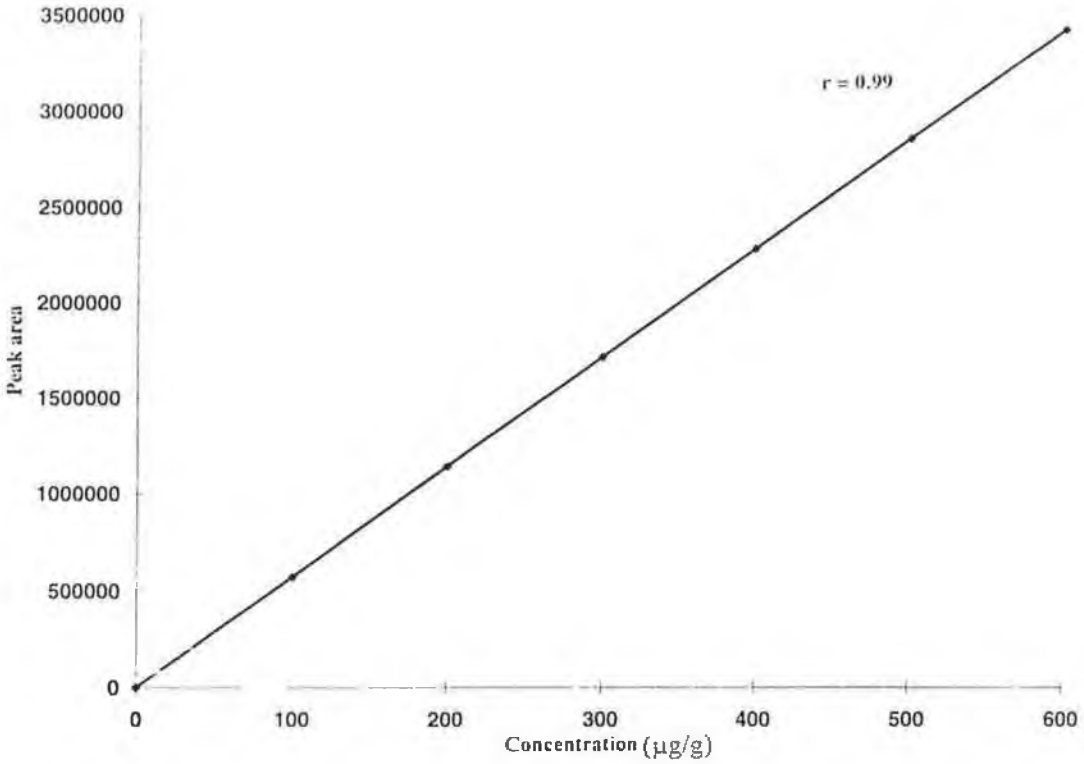


Fig. 2.9. Standard curves of (a) cholesterol and (b) cholesterol-α-epoxide.

(a)



(b)

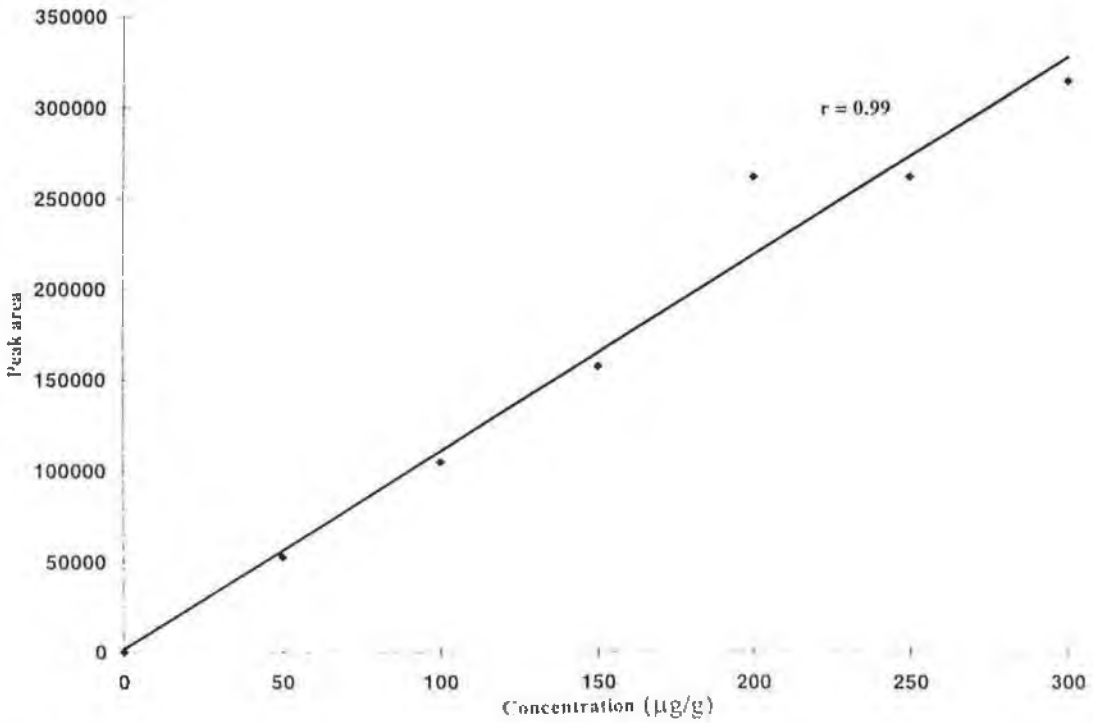
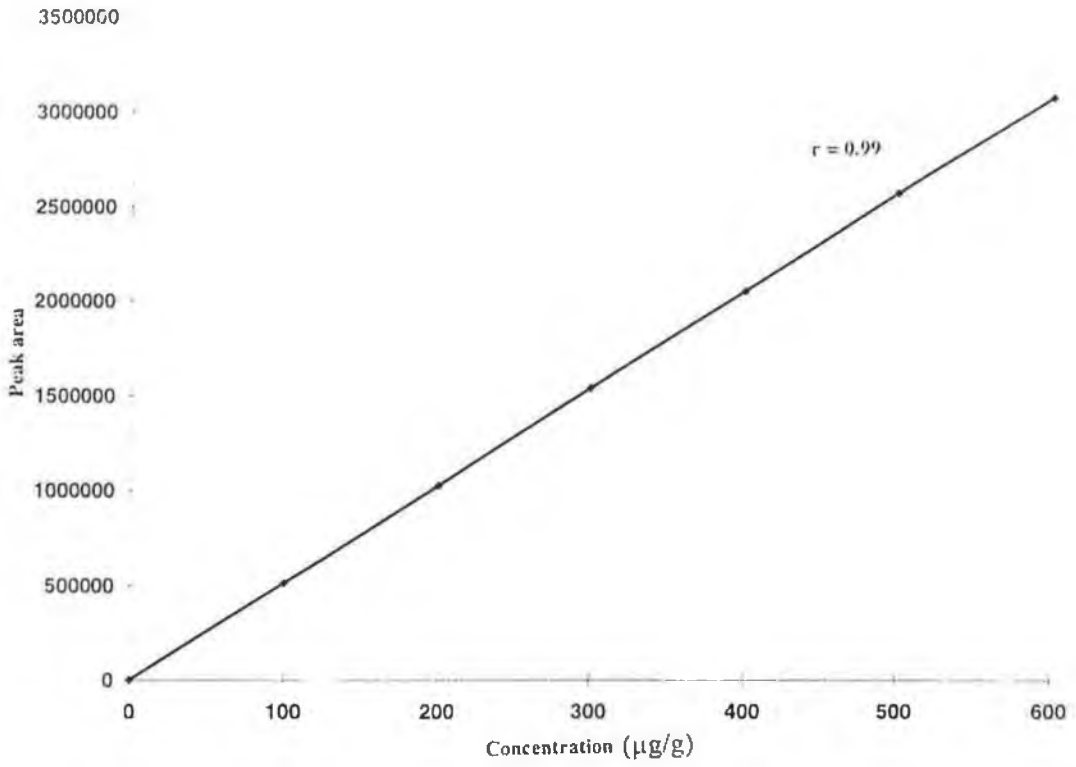


Fig. 2.10. Standard curves of (a) cholesterol-β-epoxide and (b) 25-hydroxycholesterol

(a)



(b)

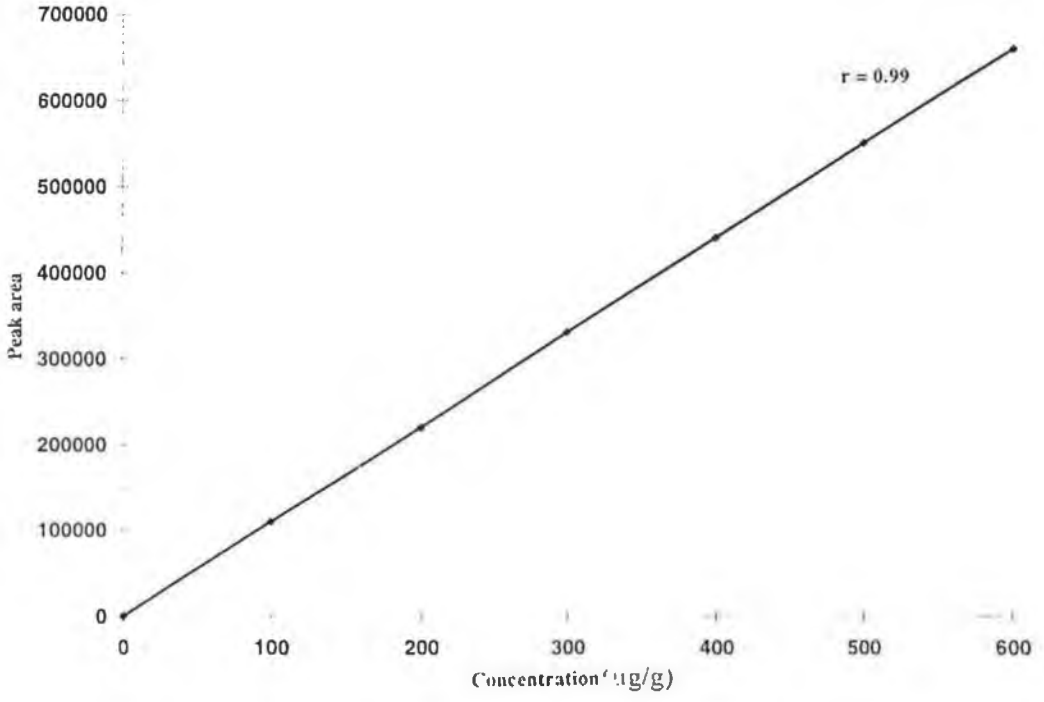


Fig. 2.11. Standard curve of (a) 7-ketocholesterol and (b) cholestanetriol

Table 2.5 Recovery of COPs from whole milk powder [a].

COPs	Initial amounts (μg/g powder)	Recovered amounts (μg/g powder)	Recovery (%)
Cholesterol	2645 [b]	572.9 ± 3.4	21.6
Cholesterol-β-epoxide	525	478 ± 20.5	91.1
Cholesterol-α-epoxide	525	482.4 ± 31.9	91.9
25-hydroxycholesterol	265	244.4 ± 16.3	92.2
7-ketocholesterol	530	481.4 ± 67.3	90.8
Cholestanetriol	575	338.9 ± 24.0	58.9

[a] n (no of analysis) = 6.

[b] 2645 μg/g = 645 μg/g spiked cholesterol + 2000 μg/g endogenous milk powder cholesterol.

% CV was lower when powder was spiked with 100 μg/g (0.75 - 2.40 %) and 1000 μg/g (0.65 - 1.98 %) (Table 2.6-2.10). Reproducibility of the assay was ascertained by triplicate analysis (on three consecutive days) of whole milk powder spiked with varying levels (10 - 1000 μg/g) of COPs. The coefficients of variation of inter-assay analysis are also shown in Tables 2.6-2.10. % CV for powders spiked with 10 μg/g COPs ranged between 4.72 - 8.33 %, while % CV ranged from 1.03 to 6.66 % for powders treated with 100 ppm and from 0.72-4.58 % when powders were spiked with 1000 ppm standard mix. The % CV for each oxide was higher when milk powders were spiked with 10 μg/g compared with 100 μg/g or 1000 μg/g of the standard mix. Willard *et al.* (1988) proposed that % CVs of less than 10 % are acceptable. The variations in % CV observed are likely to be due to analytical error, variations in GC response and/or day to day variations in sample preparation and analysis.

Table 2.6. Intra-assay and inter-assay variation following spiking of whole milk powder at 3 different concentrations of cholesterol.

	Intra-assay variation		**Inter-assay variation**	
Concentration spiked (μg/g)	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**	**Peak Area Mean ± S.D.** [a]	**C.V.(%)**
10	28978 ± 1140	3.93	31158 ± 1941	6.22
100	253687 ± 1924	0.75	351292 ± 2041	0.58
1000	1790367 ± 24110	1.34	1541505 ± 11141	0.72

[a] n (number of analysis) =3

Table 2.7. Intra-assay and inter-assay variation following spiking of whole milk powder at 3 different concentrations of cholesterol-α-epoxide.

	Intra-assay variation		Inter-assay variation	
Concentration spiked (μg/g)	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**
10	1267 ± 41	3.23	1502 ± 64	4.26
100	10024 ± 241	2.40	13983 ± 471	3.36
1000	105260 ± 1141	1.08	121481 ± 3614	2.97

[a] n (number of analysis)=3

Table 2.8. Intra-assay and inter-assay variation following spiking of whole milk powder at 3 different concentrations of 25-hydroxycholesterol.

	Intra-assay variation		Inter-assay variation	
Concentration spiked (μg/g)	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**
10	7276 ± 367	5.04	8411 ± 397	4.72
100	17724 ± 841	4.74	15564 ± 1041	6.66
1000	159878 ± 1041	0.65	183630 ± 8411	4.58

[a] n (number of analysis)=3

Table 2.9. Intra-assay and inter-assay variation following spiking of whole milk powder at 3 different concentrations of 7-ketocholesterol.

	Intra-assay variation		Inter-assay variation	
Concentration spiked (μg/g)	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**
10	7266 ± 313	4.3	5025 ± 419	8.33
100	17158 ± 447	2.60	19411 ± 921	4.74
1000	133989 ± 871	0.65	107348 ± 1001	0.93

[a] n (number of analysis)=3

Table 2.10. Intra-assay and inter-assay variation following spiking of whole milk powder at 3 different concentrations of cholestanetriol.

	Intra-assay variation		Inter-assay variation	
Concentration spiked (μg/g)	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**	**Peak Area Mean ± S.D.** [a]	**C.V. (%)**
10	7823 ± 414	5.29	3542 ± 158	4.46
100	76729 ± 943	1.20	68448 ± 711	1.03
1000	1065601 ± 21071	1.97	1172785 ± 31009	2.64

[a] n (number of analysis)=3

2.5.6 Comparison of present method with previous methods

Table 2.11 compares the present method with that of previous methods reported. The main difference is the absence of a saponification step. The methods of Sander *et al.* (1989) and Chan *et al.* (1993) are similar to the present method in that they both use the folch extraction, indirect or no saponification and an NH_2 Sep-Pak clean-up procedure. Rose-Sallin *et al.* (1995) does not extract lipids directly, but saponifies the milk powder (direct saponification) as opposed to saponification of the extracted lipids (indirect saponification). The present study used a DB-5 column in GC analysis which gave good resolution and short retention times. GC.FID and GC.MS was carried out as opposed to GC.FID only as was done by Sander *et al.* (1989) and Chan *et al.* (1993) to ensure accurate confirmation of peaks.

Table 2.11 Comparison of methods of COPs determination

	Extraction	Saponification	Clean-up	GC analysis	Confirmation
Present Study	Folch	No Saponification	NH_2 Sep-Pak	DB-5	GC.FID and GC.MS
Nielsen *et al.* (1996)	Folch	Indirect Saponification	NH_2 Sep-Pak/HPLC	DB-1	GC.MS
Rose-Sallin *et al.* (1995)	None	Direct Saponification	NH_2 Sep-Pak	DB-1	GC.MS
Chan *et al.* (1993)	Folch	No Saponification	NH_2 Sep-Pak	DB-1	GC.FID
Sander *et al.* (1989)	Folch	Indirect Saponification	NH_2 Sep-Pak	DB-1	GC.FID
Nourooz-Zadeh and Appelqvist (1988)	Hexane/Isopropanol	Indirect Saponification	C_{18}- Sep-Pak and Lipidex	DB-1	GC.MS

2.5.7 Quantification of COPs in commercial milk powders

A survey was carried out among a number of Irish milk powder manufacturers to ascertain the levels of COPs in a number of commercial milk powders. A questionnaire was distributed to Dairy Co-Operatives to obtain details of (a) type of milk powder (b) heat class (pre-heat temperature) (c) production date (d) storage temperature and (e) atomiser type (see appendix I). Table 2.12 demonstrates the levels of five COPs: 7-ketocholesterol, cholesterol-α-and β-epoxides, 25-hydroxycholesterol and cholestanetriol, expressed as μg/g in the spray-dried fresh milk powder products. COPs were not detected in low heat-treated cream, whole milk or skim milk powders. On the other hand, fresh (analysed within 1 week of manufacture) spray-dried powders from the high-heat class contained levels of 7-ketocholesterol (full cream and whole milk powder) up to 1.25 μg/g lipid, cholesterol-α-epoxide (whole milk powder) up to 1.23 μg/g lipid and cholesterol-β-epoxide (skim milk and whole milk powder) up to 1.78 μg/g lipid (Table 2.12).

Analysis of stored spray-dried milk powder products was also carried out as part of the survey, results of which are shown in Table 2.13. Stored low-heat treated whole milk powders contained higher levels of 7-ketocholesterol (12.45 ± 0.89 μg/g lipid) than the corresponding fresh powders. Low-heat whole milk powder contained quantities of β-epoxycholesterol up to 5.67 μg/g lipid. Low-heat treated full cream milk powders stored at hot room conditions (30^{0} C) also contained increased level of 7-ketocholesterol (39.10 μg/g lipid) and cholesterol-β-epoxide (4.32 ± 0.67 μg/g lipid) compared to fresh milk powders. Low-heat skim milk powder contained cholesterol-α-epoxide up to 3.51 μg/g lipid. The stored high-heat treated powders contained substantially less COPs than the low-heat treated powders (Table 2.13). The Mann Whitney test was used to analyse the significance of the results and it was found that there was a significant difference ($p < 0.001$) between low-heat and high-heat powders and no significant difference between medium-heat and high-heat powders in COPs levels. 25-hydroxycholesterol and cholestanetriol were not detected in any of the fresh or stored powders samples.

Table 2.12 COPs in spray-dried fresh [a] milk powder products. [b]

		COPs (μg/g lipid) [c]				
Product	**Pre-heating Temperature**	**7-keto**	**-α-epoxide**	**β-epoxide**	**25-hydroxy**	**Triol**
WMP *	High	0.89± 0.03	1.23±0.08	1.45± 0.06	ND	ND
WMP *	Med/high	1.11	ND	1.78± 0.03	ND	ND
WMP *	Low	ND	ND	ND	ND	ND
SMP **	High	ND	ND	1.05	ND	ND
SMP **	Low	ND	ND	ND	ND	ND
FCMP ***	High	1.25± 0.05	ND	ND	ND	ND
FCMP ***	Low	ND	ND	ND	ND	ND

[a] Analysed within 1 week of manufacture

ND: Not detected, detection limit approx. 0.1μg/g.

[b] n (number of samples) = 7

[c] Means of duplicate analysis (extraction, enrichment and GC analysis)

*** FCMP: Full cream milk powder

** SMP : Skim milk powder

* WMP : Whole milk powder.

Table 2.13 COPs in spray-dried stored[a] milk powder products. [b]

			COPs (μg/g lipid) [c]				
Product	**Pre-heating Temp.**	**Storage Temp.**	**7-keto**	**α-epoxide**	**β-epoxide**	**25-hydroxy**	**Triol**
WMP *	High	15° C	0.43± 0.03	ND	ND	ND	ND
WMP *	Medium	15° C	ND	ND	ND	ND	ND
WMP *	Low	15° C	12.45± 0.89	ND	5.67± 0.31	ND	ND
SMP **	High	15° C	ND	ND	ND	ND	ND
SMP **	Low	15° C	1.21± 0.08	3.51± 0.12	ND	ND	ND
FCMP ***	Low	30° C	39.10± 2.67	ND	4.32± 0.67	ND	ND

[a] 12 month old sample

ND: Not detected, detection limit approx. 0.1 μg/g.

[b] n (number of samples) = 7

[c] Means of duplicate analysis (extraction, enrichment and GC analysis)

*** FCMP: Full cream milk powder

** SMP : Skim milk powder

* WMP : Whole milk powder.

2.6 DISCUSSION

Retention times and relative response factors

In the present study, the complete separation of all relevant COPs in milk powders was achieved by non-polar capillary GC using a DB-5 column while MS was used to confirm their presence in whole milk powders (Fig. 2.3). Identification of COPs was achieved by comparison of retention times and by comparing mass spectra of COPs peaks with those of authentic standards (Table 2.3).

Mass spectrometric analysis of the compounds was achieved after electron impact (EI) ionization and the mass spectra of the TMS-derivatised compounds led to fragment ions that were predicted from their structure. The mass spectrum of cholesterol was characterised by a dominant ion at m/z 129 which is the base peak, and smaller ions at m/z 253 and 351. The base peak ion at m/z 129 originates from the A-ring fragmentation between C1-10 and C3-4 as previously reported (Park and Addis, 1992; Lai *et al.*, 1995a) (Table 2.3). The base peak ion of 19-hydroxycholesterol which enabled its confirmation was found at m/z 145 with smaller fragments at m/z 197 and 353. The ion at m/z 353 has previously been reported in mass spectra of 19-hydroxycholesterol (Rose-Sallin *et al.*, 1995). Cholesterol-α-epoxide showed extensive fragmentation, leaving only a few ions in the high m/z region. The base peak ion was found at m/z 135 with another ion at m/z 366. While the base peak at m/z 135 has not been reported previously, the m/z at 366 was previously found (Pie *et al.*, 1990; Rose-Sallin *et al.*, 1995). A base peak at m/z 131 was found in the mass spectrum of 25-hydroxycholesterol, which is reported to originate from α-cleavage between C24 and 25 (Park and Addis, 1992; Rose-Sallin *et al.*, 1995). No other ions were used in the confirmation of 25-hydroxycholesterol. 7-Ketocholesterol was characterised by the base peak at m/z 174 along with two further peaks at m/z 159 and 455. Finally, mass spectrometric analysis of cholestanetriol, the only cholesterol oxide among those studied to have three hydroxy groups, resulted in a base peak at m/z 445, and an ion at m/z 143. While mass spectrometric detection was carried out by a number of investigators (Nourooz-Zadeh and Appelqvist, 1988; Van de Bovenkamp *et al.*, 1988; Pie *et al.*,

1990; Rose-Sallin *et al.*, 1995 and Lai *et al.*, 1995a) only a few (Pie *et al.*, 1990; Rose-Sallin *et al.*, 1995 and Lai *et al.*, 1995a) actually reported details of the mass spectra found, hence limiting comparisons with previous findings.

Incomplete separation of some COPs or overlapping pairs, such as those of the isomeric forms of cholesterol α- and β-epoxides has been a major difficulty in the quantification of COPs by capillary GC (Fisher *et al.*, 1985). However, in the present study, good resolution of α and β-epoxides as well as cholestanetriol and 7-ketocholesterol was obtained. This was achieved by careful optimisation of the flow-rate of the carrier gas to 5 ml/min in conjunction with temperature programming to ensure complete resolution of peaks. Because of its higher concentration in samples, the bulk of cholesterol was separated from the oxysterols during solid-phase extraction. Cholesterol did not interfere with any of the oxides, as 78 % of cholesterol in milk powder extract eluted with the hexane/diethyl ether wash. This was ascertained by spiking a milk powder sample with cholesterol and monitoring its recovery. The amount spiked was 645 μg/g, and the level recovered was 572 μg/g. When the endogenous cholesterol in milk was taken into account, the recovery of cholesterol was 21.6 %. Since the cholesterol was not labelled, it was not possible to differentiate between spiked and basal cholesterol.

19-hydroxycholesterol was chosen as the IS since it has not been found in food products (Appelqvist, 1996) and it elutes close to the COPs investigated in the present study (Fig. 2.3). Relative response factors, defined as COPs response with respect to the internal standard ranged from 0.70 to 1.38 suggesting reproducible detector performance. Despite the critical role of response factors in quantitative analysis, they are seldom described in the literature. Nourooz-Zadeh and Appelqvist (1987) applied the same response factors, i.e. 1.0 to all COPs, citing Park and Addis (1985a), which may have resulted in over-estimation of the level of COPs present, since no correction factor (response factor) was used in their estimation.

Recovery of cholesterol and cholesterol oxides

Given that the concentration of cholesterol in milk lipids is 2 mg/g and that less than 1 ng/g of cholesterol is normally oxidised in milk (Nielsen *et al.*, 1995), the large number of steps in the analytical procedure for COPs hampers their successful analysis. Moreover, oxysterols have a polarity very similar to the polarity of the triacylglycerides (Nielsen *et al.*, 1995), which account for 97-98 % of the milk lipids. Separation and quantification of oxysterols in dairy products are therefore hampered by the combination of low concentration and similarity in polarity with the lipid matrix. The very different levels of oxysterols that have been reported for dairy products of similar type from laboratories using different analytical methods may be traced to differences in these methods and may not reflect a real difference in the content of oxysterols. This lack of confidence in some of the reported analytical results is further substantiated by the limited validation of the methods employed (Nielsen *et al.*, 1995).

Because of the low concentration of COPs in foods, most attempts to measure these products require isolation and concentration steps to precede the actual determination. Previous workers have employed saponification of the extracted lipids with hot alkali as an important part of the enrichment procedure. This step separates the bulk of the lipids from the sterol residue and also converts esterified cholesterol to free cholesterol. However, some important COPs, notably 7-ketocholesterol have long been known to be unstable to hot, aqueous alkali (Chicoye *et al.*, 1968; Finocchiaro and Richardson, 1983; Finocchiaro *et al.*, 1984). A number of investigators (Smith, 1981; Van de Bovenkamp *et al.*, 1988 and Appelqvist, 1996) have reported the artefactual formation of COPs during the course of the saponification procedure. It has previously been reported that chromatographic techniques can be applied to separate COPs not only from each other but also from the large amounts of interfering triglycerides, cholesterol, phospholipids and other matrix lipids present (Schmarr *et al.*, 1996). The similarity between lipid class structures requires a highly selective extraction procedure. The lipid extract containing 50 μg of 19-hydroxycholesterol, was transferred to an NH_2 Sep-Pak

cartridge. The column was then washed with 10 ml hexane and 15 ml hexane : diethyl ether (95 : 5 (v/v)) to elute most of the trigylcerides from the column, thereby separating the COPs from the triacylglycerols. The remaining triglycerides and cholesterol were eluted by washing the column with 25 ml hexane : diethyl ether (90 : 10 (v/v)) and 15 ml of hexane : diethyl ether (80 : 20 (v/v)). The COPs were eluted off the column with 10 ml acetone and evaporated to dryness under nitrogen.

In the present study, the greatest loss was incurred at the clean-up stage of the procedure (Table 2.4). This may have been due to incomplete solvation of the sorbent bed; sample breakthrough; inadequate numbers of washes in the clean-up stage leading to increased potential for loss or inconsistent flow-rate through the column. Attempts made to improve the procedure involved the following precautionary steps: the use of nitrogen to retard oxidation, aluminium foil to eliminate light and oxygen; maintenance of the temperature of the water in the evaporation stage at 35° C; maintenance of a constant flow through the Sep-Pak cartridge, while not allowing the pressure to exceed 5-10 psi and finally performing all analyses on the same day. These precautionary steps reduced the losses incurred by approximately 10 %, resulting in recoveries of approximately 90 %. Lai *et al.* (1995a) also used a vacuum manifold to maintain a constant flow-rate in Sep-Pak clean-up of samples. Whole milk powder which was not subjected to clean-up with a Sep-Pak column showed a number of unidentified peaks at the start of the chromatogram, which may have been triglycerides and/or phospholipids. By contrast, these peaks were absent from powder samples which were subjected to the solid-phase extraction procedure (Fig. 2.8). These peaks masked the COPs of interest, and hence the solid-phase Sep-Pak extraction procedure was employed as a routine step in COPs analysis of whole milk powder. The Sep-Pak clean-up procedure may also have aided in prolonging the lifetime of the GC column used for COPs analysis, as the sample which did not undergo a clean-up could result in column overload. The advantages of solid-phase extraction over traditional sample preparation methods include increased speed and simplicity, reduced solvent usage and disposal costs and improved selectivity. Analyte breakthrough is one of the potential problems associated with solid-phase extractions. This occurs when the

column is overloaded, when solvents have not been properly selected, when pores become blocked or when flow through the column is too fast. In the present study, a vacuum manifold was used to increase the speed of elution and care was taken not to allow the pressure to exceed 5-10 psi. Care was also taken to ensure that Sep-Paks from the same batch were used for a given set of samples, as batch-to-batch variability in commercial solid-phase extraction cartridges is frequently observed (Blevins *et al.*, 1990). Differences have been reported in stationary phase loading due to the extent of end-capping of bonded phases from different sources (Blevins *et al.*, 1990). These observed differences may be due to differences in manufacturing methods between vendors. One major disadvantage of Sep-Pak columns is that separations on solid-phase extraction columns cannot be monitored, and hence method development can be difficult and lengthy. This could be alleviated by using TLC to confirm solid-phase extraction separation or to identify components in a given eluent.

TMS ether derivatives of COPs were prepared by redissolving COPs in BSTFA. Derivatisation using BSTFA was necessary in order to prevent interaction of active hydrogens from the sterol oxides with metal wall surfaces or the stationary phase itself (Csallany *et al.*, 1989). The BSTFA solution was heated at 120° C for 30 min, as these conditions were previously reported to result in reproducible derivatisation. Poor reproducibility resulted when COPs were heated at lower temperatures (80° C) and for longer periods (2 h) (Nawar *et al.*, 1991).

Validation of cholesterol oxide analysis

Once the chromatographic procedure was optimised for separation of COPs, the entire method was validated prior to milk powder analysis. Linearity of a method is its ability to produce a response that is proportional to the concentration of an analyte within a given range. The standard procedure is to analyse a set of standards and subject the results to linear regression analysis. The correlation coefficient is then used as an estimate of 'best fit'. Linear responses were recorded for each COPs, with correlation coefficients of approximately 0.99 which indicates that the

method was linear in the range 0-600 μg/g using GC.FID (Figs. 2.9-2.11) and 0-1000 μg/g using GC.MS (Appendix II).

To assess recovery, varying amounts of COPs were added to whole milk powder which were processed through the entire procedure and finally analysed by GC. The amount recovered was compared with the COPs standards which had been derivatised and analysed directly by GC. By taking the areas of the standards that were directly injected onto the column as 100 %, the recoveries of COPs from milk powder samples after extraction, clean-up and derivatisation were calculated accordingly. The recoveries obtained were greater than 90 %, except for cholestanetriol (Table 2.5). Full recovery was not obtained possibly because of the trace amounts of COPs present in milk powders and also because of possible interference from the bulk lipids in milk (Csallany *et al.*, 1989). One reason for the low recovery of cholestanetriol may be adsorption, since cholestanetriol has three hydroxyl groups which may adsorb to glass and other reactive substances (Granelli *et al.*, 1996). Pie *et al.* (1990) suggested that poor recovery of cholestanetriol was possibly due to the fact that it is poorly extracted from silica. Another reason for poor recovery is aging GC columns, with older columns reported to give rise to peak broadening, peak tailing, poor resolution and consequently poor recoveries (Appelqvist, 1996). Pie *et al.* (1990) demonstrated that 20-hydroxycholesterol and cholesterol epoxides were slightly overestimated possibly due to the fact that they were extracted more effectively from silica than the internal standard, 19-hydroxycholesterol. The percent recoveries of COPs reported in the literature range from 23.6 % (Higley *et al.*, 1986) to close to 100 % (Nielsen *et al.*, 1995; Rose-Sallin *et al.*,1995). Specific flow-rates of solvents have not been been reported in other studies in which solid-phase extraction was used to isolate COPs from other lipids and/or cholesterol (Lai *et al.*,1995a). Therefore, the large variations among the recoveries of COPs reported in the literature may, in part at least, be attributed to inconsistent flow-rates (Lai *et al.*,1995a). The higher recoveries obtained in recent years may be a reflection of our better understanding of separation techniques in analytical chemistry.

In the present study, repeatability of the GC procedure for quantification of cholesterol and its oxides was achieved by triplicate injections of three concentrations of each of the oxides on the same day (Tables 2.6-2.10). Coefficients of variation of less than 5.3 % were obtained indicating acceptable repeatability (Willard *et al.*, 1988). The reproducibility of the present method was ascertained by running cholesterol and its oxides through the entire procedure on 3 successive days. The coefficient of variation were less than 8.33 %, which again indicated that this method was reproducible and suitable for analysis of COPs in milk powders (Willard *et al.*, 1988; Schmarr *et al.*,1996). Similar % CV values (2-7 %) were reported by Schmarr *et al.* (1996). Rose-Sallin *et al.* (1995) reported higher coefficients of variation (up to 34 %) and attributed this to decomposition of oxides during the injection step and the closeness of the level of COPs found in the milk powders with the limit of detection of their method (10-100 ng/g).

The limit of detection of the method was found to be 0.1 μg/g, defined as the least detectable concentration of sample or the concentration where the signal height is three times that of the noise (Nielsen *et al.*, 1995). Reported limits of detection for COPs range from 0.01 (Rose-Sallin *et al.*, 1995) to 0.1 μg/g (Van de Bovenkamp *et al.*, 1988; Nourooz-Zadeh and Appelqvist, 1988). The variation in the levels reported may be due to the differences in the chromatographic methods used for COPs detection.

Quantification of COPs in commercial milk powders

In Ireland, there is an average of 36,000 tonnes of whole milk powder produced every year by an average of 12 spray-drying plants. In the analysis of freshly made commercial spray-dried whole milk, cream and skim milk powders, no COPs were detected at the limit of detection (0.1 μg/g) for low- and medium-heat powders. On the other hand, fresh spray-dried whole and skim milk powders from the high-heat category showed quantifiable levels of 7-ketocholesterol and cholesterol-β-epoxide (Table 2.12). Analysis of stored spray-dried milk powder products was also carried out as part of the survey, and increased levels were found in the stored powders

(Table 2.13). The results of the present survey compare favorably with those of Nourooz-Zadeh and Appelqvist (1988) who carried out a survey amongst the 14 spray-drying plants and 2 roller-drying plants in Sweden. They found that in the analysis of freshly produced spray-dried cream, whole milk and skim milk powder products, no COPs were detected for low- and medium-heat powders and similarly fresh roller-dried whole milk powder contained no quantifiable amount of COPs. They also found that fresh spray-dried whole and skim milk powders from the high-heat category showed quantifiable amounts of the hydroxycholesterols, the epimeric epoxides and cholestanetriol.

2.7 CONCLUSIONS

A method was successfully established and validated for reliable quantification of COPs in whole milk powders. Lipid extracts of milk powders were successfully analysed for their content of 5 COPs using a Folch extraction and solid-phase extraction procedure. COPs were separated from triglycerides on an NH_2 Sep-Pak cartridge by elution with hexane : diethyl ether (95:5 v/v; 90:10 v/v and 80: 20 v/v), and then separated from each other and identified using GC.FID. Confirmation of structural identity was carried out using GC.MS. The limit of detection of the method was 0.1 μg/g. The method was suitable for analysis of COPs in milk powders with levels detected being consistent with those found previously.

CHAPTER 3

EFFECT OF ANIMAL FEEDING REGIMEN ON WHOLE MILK COMPOSITION AND OXIDATIVE STABILITY OF WHOLE MILK POWDER

3.1 INTRODUCTION

It has frequently been reported that dairy product manufacturers in Ireland as well as in parts of Australia, New Zealand and the United Kingdom experience difficulty in producing high quality dairy products consistently thoughout the year. The problems are seasonal and believed to be related to changes in milk characteristics associated with stage of lactation, level of feeding and diet quality (Cullinane *et al.*, 1984; Grandison *et al.*, 1984, O'Keefe, 1984; Kefford *et al.*, 1995). Differences in husbandry practices and calving patterns within and between countries may also lead to variations in the composition of milk supplied to processing units (Phelan *et al.*, 1982).

Major difficulties exist with the processability of a seasonal milk supply (Phelan *et al.*, 1982). Late-lactation milk, for example, has serious limitations as a raw material for processing. The efficiency of centrifugal separators is much reduced due to the greater proportion of small fat globules (< 1μm) in late-lactation milk and this may present a difficulty in conforming with the EC limit of 1.25 % fat in skim milk powder for intervention (Commission Regulation, 1978). In addition, the low thoughput of milk from cows in late-lactation poses efficiency problems for equipment that is designed to handle larger volumes associated with a peak supply. Increased susceptibility to agitation and air incorporation can lead to a greater disruption of the fat globule membrane which is weaker in late-lactation. This disruption causes an increase in free-fat levels in milk. Furthermore, particles of the fat acquire a new protein membrane and form smaller globules which are difficult to separate (Phelan *et al.*, 1982). A higher content of copper is also problematic as it contributes to the susceptibility of late-lactation milk to fat oxidation (Van Mils and Jans, 1991). In Ireland, milk for manufacturing purposes is produced exclusively by Spring calving herds. Thus, heat-stable milk powders can only be manufactured from March to October (Phelan *et al.*, 1982). Although this practice takes maximum advantage of grass as a cheap feed, it may nevertheless result in extremes of milk volume and composition as the digestibility and quantity of the herbage can vary considerably between March and October.

In view of the difficulties in relying on grass only as a feed source, intense efforts in recent times have been made to improve milk composition by altering the quality of the animal diet (Astrup *et al.*, 1980, Palmquist *et al.*, 1993, Grummer, 1991, Kefford *et al.*, 1995, Murphy *et al.*, 1995). Although the relationship between feed constituents and milk composition is complex, animal diet quality has been reported to exert a significant impact on milk composition (Sutton, 1989).

Milk composition varies in response to certain nutritional manipulations such as severe underfeeding (Sutton, 1989), concentrate supplementation (Rook, 1971; Rook *et al.*, 1992) and frequency of feeding (Grummer, 1991). The earliest experiment using animal diet as a method of manipulating milk composition was in 1937 when Powell found a direct correlation between roughage intake and the composition of milk (Powell, 1938). Powell (1938). also considered that there was a definite correlation between the activity of the microflora of the rumen and the composition of milk. Since then, many dietary components have been shown to be capable of altering the concentration of fat or protein in milk.

Most of the attention paid to milk composition has focused on milkfat content as this is most sensitive to dietary influences and of the thee major solids (fat, protein and lactose) in milk, it can be changed over the widest range (Sutton, 1989; Palmquist *et al.*, 1993). Many studies have shown that it is possible to alter the fatty acid composition of milkfat by feeding different fats and oils (Banks *et al.*, 1984; De Peters *et al.*, 1985; Murphy *et al.*, 1990, 1995). Feeding full fat soyabeans or rapeseeds containing unsaturated 18-carbon fatty acids, to lactating cows resulted in a softer fat (Murphy *et al.*, 1995). The unsaturated fatty acids are hydrogenated by microflora in the rumen to stearic acid which is then absorbed from the intestine and converted to oleic acid in the mammary gland by an intramammary stearic acid desaturase (Palmquist *et al.*, 1993). Thus, increasing the supply of stearic acid to the mammary gland results in increased levels of oleic acid in the milkfat thereby resulting in a softer fat (De Peters *et al.*, 1985; Grummer, 1991; Murphy *et al.*, 1995).

Effects of dietary protein on milkfat composition are less well documented (Palmquist *et al.*, 1993). Higher protein intake increased mobilization of adipose tissue in early lactation (Oldham, 1984), which led to an increase in the proportion of long-chain fatty acids in milk. Energy intake influences milkfat composition in a number of ways. When an animal is in positive energy balance, increased synthesis of short-chain fatty acids occurs. By contrast, when an animal is in negative energy balance the reduced dietary supply of acetate and glucose reduces synthesis of short-chain fatty acids causing a consequent increase in mobilization of fatty acids from adipose tisssue (Luick and Smith, 1963; Palmquist *et al.*, 1993). Changes in milkfat composition are accompanied by other changes including decreased milk protein concentration and increased milk urea concentration (non-protein nitrogen) (Palmquist *et al.*, 1993). Milk protein accounts for approximately 25 % of total milk solids and is a more valuable component of milk than fat (Murphy and O'Mara, 1993). Increasing the amino acid supply to the mammary gland is the basis for most dietary manipulations to increase protein concentration or yield. Dietary protein does not affect milk protein concentration (Butler *et al.*, 1983; Hettinga, 1989). However, feeding higher levels of concentrate supplement, thereby increasing the plane of nutrition of the cow, has been reported to increase both milk protein concentration and yield (Rook *et al.*, 1992). The inclusion of lipid in either a rumen-protected or unprotected form has resulted in a decrease in milk protein concentration but not in protein yield except at high levels of inclusion (Murphy and Morgan, 1983; Banks *et al.*, 1984).

Few attempts have been made to segregate and quantify the relative influences of each of these factors on product quality and performance. Recently, however, Kefford *et al.* (1995) showed separate effects of diet and stage of lactation on protein and fat contents of milk used for Cheddar cheese manufacture in Australia.

The absolute criterion for suitability of milk for processing is stability during manufacture and storage, often over extended periods, of high-quality dairy products (Phelan *et al.*, 1982). A major aspect of the quality of whole milk powder is its sensory quality which declines upon storage as a result of 'off-flavour' development

caused by autoxidation. While there have been a number of systematic studies carried out on the influence of animal diet quality on lipid oxidation (Buckley and Connolly, 1980; Atwal *et al.*, 1991; Monahan *et al.*, 1990; 1992; Li *et al.*, 1996) there have been no known studies reported on it's influence on cholesterol oxidation in whole milk powder.

The second stage of the research investigated the influence of animal feed quality on whole milk powder performance during storage with particular regard to the appearance of cholesterol oxides in these powders. The term animal feeding regimen as used thoughout this thesis refers to the feeding regimes employed. Cows were fed either a restricted grass supply or an amount of concentrate which supplemented the standard grassland management system. The concentrate furnished extra protein, oil, crude fibre, minerals and vitamins including α-tocopherol. The feeding regimens were compared with respect to their influence on lipid and cholesterol oxidation in whole milk powders. Milk from both herds was taken for powder production at intervals corresponding to early, middle and late-lactation. In order to segregate the effects of processability from those of animal feed quality and stage of lactation, all powders were manufactured using a low pre-heat treatment prior to spray-drying. The powders were sachet-packed in foil-lined paper bags and stored at 15° C for 12 months. Peroxide values (PVs), thiobarbituric acid reactive substances (TBARS) and cholesterol oxidation products (COPs) were used as indices for monitoring oxidation occurring in the powders during storage.

3.2 AIMS

To investigate the influence of nutrition on the composition of milk and resultant whole milk powders.

3.3 OBJECTIVES

- To investigate the influence of animal feeding regimen on composition of milk and powder products

- To determine the oxidative stability of whole milk powder, when sachet-packed and stored at ambient temperature.

3.4 EXPERIMENTAL

3.4.1 Reagents

DL-α-Tocopherol (E307) and Ronoxan D20 were received as gifts from Roche Products Limited, Hertfordshire, U.K. All other reagents, including thiobarbituric acid and ammonium thiocyanate, were Analar grade and were purchased from BDH (Poole, Dorset, UK). Chloroform and methanol were HPLC grade, while all other solvents were Analar grade and were purchased from Labscan Ltd. (Stillorgan, Co. Dublin). Kjeltabs (5 g Potassium Sulphate K_2SO_4,, 0.5 g Copper (II) Sulphate, $CuSO_4$) were purchased from Alkem, Little Island, Co. Cork.

3.4.2 Feeding regimen

Thirty two Spring-calving Friesian cows were allocated to two herds (n = 16) on the basis of calving date and milk yield one week before initiation of the experiment, until the end of the feeding experiment which lasted 26 weeks.

Dietary Treatments:

R = Grass-fed.

S = Grass + concentrate-fed

Herds R and S were stocked at 4.0 cows/ha and 3.57 cows/ha, respectively. Herd S cows received 3 kg concentrate/cow/day. The herds were grazed separately with a residence time of 3 days/plot. The concentrate furnished 186 g/kg protein, 38 g/kg lipid, 100 g/kg crude fibre and 91 g/kg ash. The vitamin E concentration of the concentrate was 7,500 IU/ton and supplementation was therefore 22.5 IU vitamin E/cow/day.

Composition of the concentrate

375 kg/ton unmolassed sugar beet pulp

494 kg/ton corn gluten feed

99 kg/ton rapeseed meal

10 kg/ton tallow

7 kg/ton salt

6 kg/ton limestone flavour

7 kg/ton dicalcium phosphate

2 kg/ton calcined magnesite

10 kg/ton minerals and vitamins

Minerals and Vitamins

80 g/ton Manganese oxide MnO_3

150 g/ton Copper Sulphate $CuSO_4$

118 g/ton Zinc Oxide ZnO

12 g/ton Potassium Iodide KI

4 g/ton Sodium Sulphate Na_2SO_4

12 g/ton Cobalt Sulphate $CoSO_4$

7.5 mIU/ton Vitamin A

1.5 mIU/ton Vitamin D_3

7,500 IU/ton Vitamin E

The concentrate supplementation (kg/day) was as follows:

Herd	Apr 1-Apr 30	May 1-Sept 14	Sept 15-Oct 31
R	0	0	0
S	4	3	4

The stocking rates (cow/ha) were as follows:

Herd	Apr 1-June 20	June 21-Aug 20	Aug 21-Oct 31
R	0.40	0.50	0.80
S	0.45	0.55	0.90

The land allocation (ha) was as follows:

Herd	Apr 1-June 20	June 21-Aug 20	Aug 21-Oct 31
R	2.62	3.33	5.33
S	3.00	3.60	6.00

The target post-grazing sward heights (cm) were as follows:

Herd	Height
R	< 5.0
S	6.0-8.0

3.4.3 Milk collection, storage and transport

Milk samples were pooled and bulk milk samples from the 2 herds were pumped directly into each of two 680 litres insulated tanks. The tanks were cooled to 4° C by a chilled water system. Evening milk was pumped into each of the 2 tanks and stored overnight, after which morning milk was then added. The milk was sampled on Tuesday evening and Wednesday morning for each week. Powder was made after standardisation of the milks.

3.4.4 Manufacture and storage of whole milk powder

The whole milk powders used in this work were produced in the Moorepark pilot scale milk processing plant at intervals over a 12 month period. Milk powder manufacture required initial standardisation of milk, resulting in a powder containing 26 % fat. Only the low-heat powders were used in this study.

3.4.4.1 Standardisation of milk

Pearson's square was used to assess the amount of skim milk which should be added to the whole milk to yield a powder with 26 % fat in dry matter (Phelan *et al.*, 1982). The Pearson's square method is a diagrammatic method of reducing the risk of error in the calculation of proportions of 2 ingredients to be used to give a desired end-product (Varnam and Sutherland, 1994).

3.4.4.2 Spray-drying of whole milk powder

A flow-chart of the manufacture of whole milk powder is shown in Fig. 3.1. The standardised milk (250 L) was pre-heated, using an APV Paraflow plate heat exchanger (Type HX), fitted with a 15 s holding tube. The pre-heated milk was concentrated using a single-effect, recirculation type, Anhydro falling film evaporator (Type Lab. 3).

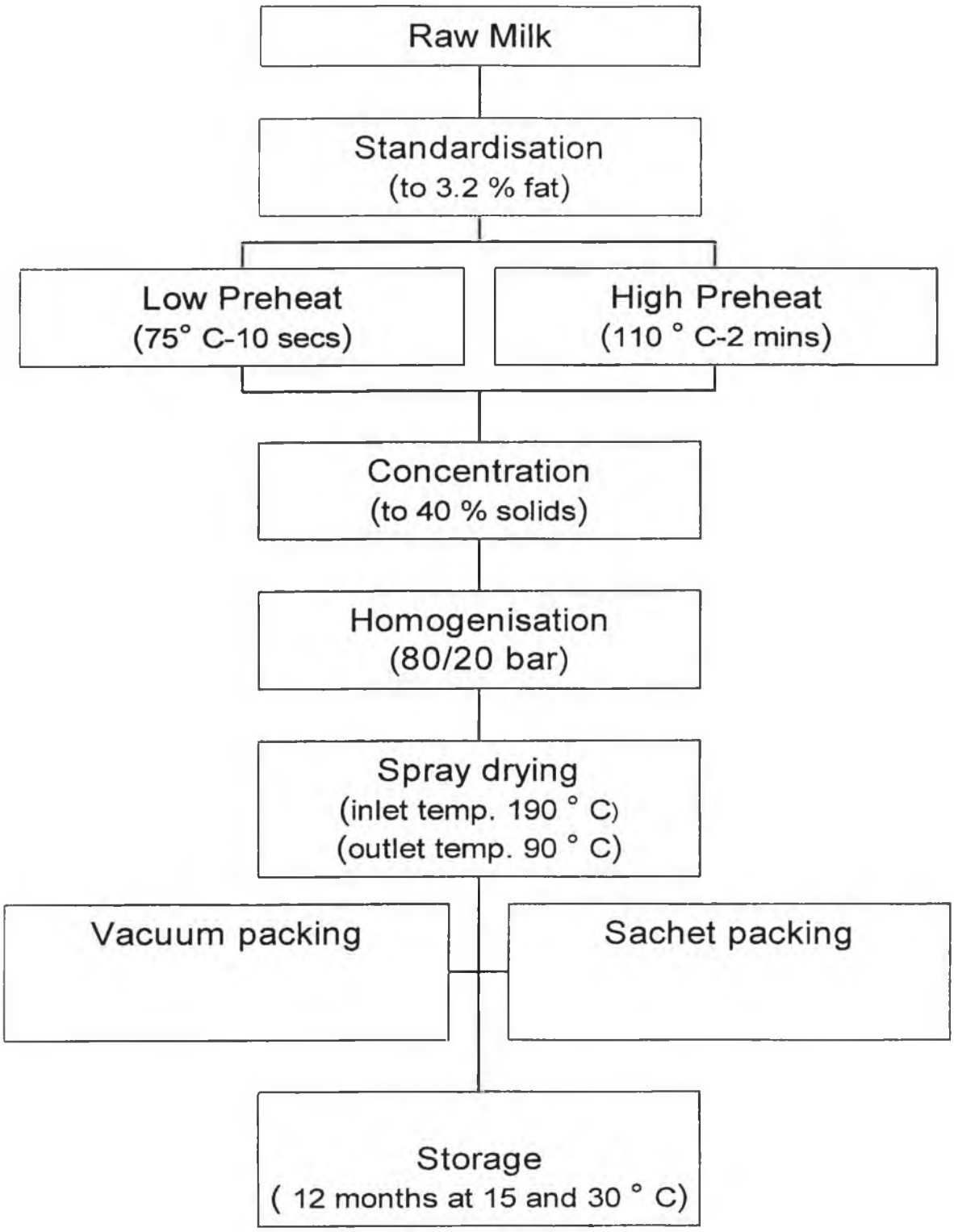


Fig. 3.1 Flowchart of low-heat and high-heat whole milk powder manufacture and storage in the present study.

The concentrate was homogenised (two-stage) using an APV Manton Gaulin homogeniser (Type KF 3). The homogenised concentrate was spray-dried using an Anhydro single-stage drier (Type 1 KA) equipped with a centrifugal atomiser. The powder was collected in a stainless steel bin at the base of the powder recovery cyclone and transferred to foil sacks. It was then allowed to cool in the sealed sacks to ambient temperature before packing.

Processing conditions were as follows:

Milk pre-heated at 75° C for 10 sec (low-heat powder) and 110° C for 2 min (high-heat powder)

Total solids of the concentrate was 40 - 42 %

Homogenisation pressure at 80/20 bar and temperature at 60° C

Spray-dryer inlet air temperature 200° C

Spray-dryer outlet air temperature 90° C

3.4.4.3 Packing of whole milk powders

Sachet packed powders were packed in aluminium-lined sachet bags and sachet sealed while the vacuum packed powders were packed into laminated aluminium bags and vacuum sealed using a Webomatic Vacuum Packer.

3.4.4.4 Storage of whole milk powders

Storage stability trials were carried out at 15° C and 30° C, for up to 12 months. Powders were stored in a walk-in room with artificial lighting, with temperature control to ± 2° C.

3.4.5 Compositional analysis of standardised milk

3.4.5.1 Compositional analysis

The protein, fat and lactose contents of milks were analysed as outlined by Phelan *et al.*, (1982) using the fully automated Milkoscan Infa-Red Analyser (Foss Electric, Denmark)

3.4.5.2 Determination of fat content in milk

The fat content of fluid milk was analysed according to the Rose-Gottlieb method (IDF, 1987). A 1 ml aliquot of milk (35 - 40° C) was added to 2 ml ammonia (25 % w/v) in a Mojonnier fat extraction flask and mixed thoroughly. 10 ml ethanol (94 % v/v) and 2 drops Congo Red indicator dye were then added and mixed gently. 25 ml petroleum ether was added, and the mixture allowed to stand for 30 s before centrifugation at 600 g for 2 min. The supernatant was decanted into a previously weighed round bottomed flask to which 15 ml diethyl ether and 15 ml light petroleum ether were added. This extraction was repeated twice. The final supernatant was added to the round bottom flask and its entire contents were heated at 102 ± 2° C for 1 h, allowed to cool to room temperature and the weight recorded. The fat content, expressed as a % by weight, was recorded as the difference in weight between the final weight of the round bottomed flask and its initial weight.

3.4.5.3 Fatty acid analysis

3.4.5.3.1 Milkfat extraction

Milk was cooled to approximately 4° C and a 10 ml aliquot centrifuged at 1000 g for 10 min. The extracted cream was then heated to 60° C and centrifuged at 1000 g for a further 10 min.

3.4.5.3.2 Milkfat methylation

100 mg of extracted milkfat was dissolved in 5 ml n-heptane and 0.5 ml 2 M methanolic KOH added. After 5 min, the solution was centrifuged at 1000 g for 3 min, and the supernatant removed for GC analysis.

3.4.5.3.3 Fatty acid analysis

A 100 µl aliquot of supernatant was added to a 2 ml chomatography vial containing 0.6 ml heptane, and 1 µl injected onto a Pye-Unicam 204 GC.FID. A packed glass column (10 % ethylene glycol adipate on a Q 10/120 mesh (Analabs, North Haven, CT, USA)) with dimensions of 2.15 m x 2 mm i.d. was used for fatty acid analysis. The N_2 carrier gas had a flow-rate of 20 ml/min. The injector temperature was 200° C and the detector temperature 250° C. The column was temperature programmed from 60° C, with a hold time of 2 min, to a final temperature of 200° C with a hold time of 32 min, at a rate of 16° C/min. Results were expressed as g fatty acids as methylester /100 g of total milk fatty acid methyl esters (FAME).

3.4.5.4 Determination of nitrogen fractions of milk by Kjeldahl analysis

Samples were heated to approximately 40 ± 2° C for 30 min and then cooled to 20 ± 2° C in an ice-bath.

3.4.5.5 Non protein nitrogen (NPN)

Protein was precipitated from 20 g of milk sample using 12 % (w/v) trichloroacetic acid (TCA) and the solution was then filtered though a Whatman No. 42 filter paper. The nitrogen content in 20 g of the filtrate was analysed using the Kjeldahl method (IDF, 1993).

10 ml concentrated sulphuric acid and one catalyst tablet (0.5 g $CuSO_4$ and 5 g K_2SO_4) were added to each tube. The tubes were heated on a digestion block at a low initial temperature (180 - 230° C), to control foaming. After approximately 30 min, the temperature of the digestion block was increased to 400-430° C. Digestion was allowed to continue until the contents of the tubes were clear green, and then for a further 1.5 h. 50 ml distilled water was then added to the cooled digest, which was then distilled into boric acid.

A conical flask, containing 50 ml 4 % boric acid was placed in the distillation unit, and the digestion tube attached. 55 ml 50 % (w/v) sodium hydroxide (NaOH) was dispensed into the tube. The ammonia, liberated by the addition of NaOH, was then

steam distilled and 200 ml distillate collected and titrated with 0.02 M HCl to pH 4.6.

3.4.5.6 Non casein nitrogen (NCN)

NCN was analysed according to the Kjeldahl method (IDF, 1993). 30 g of milk was added to 50 ml of distilled water at 50° C and 3 ml 10 % (w/v) TCA was added. The solution was allowed to stand for 10 min at room temperature before 3 ml of 1 M sodium-acetate was added and the solution made up to 100 ml with distilled water. This was then filtered though Whatman No. 42 filter paper.

3.4.5.7 Total nitrogen (TN)

Total Nitrogen was analysed in 2 g of the milk sample using the Kjeldahl method (IDF, 1993) and 10 ml water used as a blank.

3.4.5.8 Calculations

Total Nitrogen

$$\% TN = (1.4 \times (v1 - v0) \times C)/W$$

No protein content

$$\% NPN = (1.4 \times (v1 - v0) \times C \times 0.988)/W$$

No casein nitrogen content

$$\% NCN = (1.4 \times (v1 - v0) \times C \times 0.985)/W$$

Where: v1 = Sample titre (ml)

v0 = Blank titre (ml)

C = Molar concentration of the acid used for titration (0.02 ± 0.0005 M)

0.988 = Correction factor for 20 g of milk sample analysed (NPN).

0.985 = Correction factor for 30 g of milk sample analysed (NCN)

W= Weight of milk sample taken (g)

Calculations of crude total protein content

$$\% TP = \% TN \times 6.38$$

Calculation of casein protein

$$\% CP = (\% TN - \% NCN) \times 6.38$$

Calculations of casein number

$$CN = (\% \text{ Casein protein} / \% TP) \times 100$$

Calculations of whey protein content

% WP=(% NCN - % NPN) x 6.38

3.4.6 Whole milk powder analysis

3.4.6.1 Moisture analysis

Whole milk powder samples were analysed for moisture content by drying to 120° C for 10 min in a Mettler LP 16 (Mason Technology, Switzerland).

3.4.6.2 Determination of fat content in whole milk powder

The fat content of a 10 % (w/v) solution of whole milk powder was determined according to the Rose-Gottlieb procedure, as outlined in section 3.4.5.2.

3.4.6.3 Determination of undenatured whey protein nitrogen

The undenatured whey protein nitrogen index was determined by the method outlined in ADMI (1971). Whole milk powder was first reconstituted (2.7 g/20 ml) and 8 g NaCl was then added and the mixture incubated at 37° C for 30 min. The mixture was shaken 8-10 times during the first 15 min, and then filtered, without cooling though Whatman No. 42 filter paper. To 1 ml of the filtrate was added 10 ml of saturated NaCl solution (1 kg/l) which was then acidified with 30 µl 1 N HCl and the transmission recorded at 420 nm within 5 - 10 min. Undenatured whey protein was expressed as mg undenatured whey protein/g whole milk powder.

3.4.6.4 Determination of peroxide values in whole milk powder

The peroxide value (PV) of milkfat isolated from milk powder was determined using the ferric thiocyanate method, as outlined by (IDF,1991). Milkfat was extracted as outlined by Murphy (1978): 35 ml of reconstituted powder (13 g/90 ml water) and 11 ml of the de-emulsification reagent (30 g Triton X-100 and 70 g of sodium hexametaphosphate/l) were placed into narrow necked fat separation tubes (AGB Ltd., Dublin) and mixed thoroughly. The tubes were placed in a boiling water-bath and the contents were agitated after 5 and 10 min. The tubes were centrifuged in a Gerber centrifuge for 1 min. Sufficient de-emulsification reagent was then added to bring the fat column up the narrow neck of the tube and the sample was centrifuged

again for 1 min. The fat was transferred from the tube using a Gilson pipette. If the fat solidified before being transferred, the tube was put back into the heated water-bath for a further 3 - 5 min to liquify the fat once again. 9.6 ml chloroform : methanol (70:30 v/v) and 0.05 ml 0.5 M ammonium thiocyanate were added to 0.3 g of the isolated milkfat samples and the resultant solutions vortexed. Subsequently, 0.05 ml 3 M iron (II) chloride was added and the solution incubated for 10 min at room temperature. Iron (III) Chloride was used as a standard. Absorbance at 500nm was recorded and results expressed as mEq O_2/kg fat.

3.4.6.5 Determination of thiobarbituric acid reactive substances in whole milk powder

Secondary lipid oxidation products were measured by the 2-thiobarbituric acid (TBA) method of Tarladgis *et al.* (1960) using a Buchi steam distillation apparatus (Model VD 100). 7 ml 3 N HCl were added to a dispersion of whole milk powder (15 g in 75 ml) in cold distilled water. 1 drop of silicone antifoam agent was added and the contents mixed and subsequently distilled to yield 50 ml distillate. A 20 ml aliquot of distillate was added to 2 ml 0.02 M TBA (in 90 % glacial acetic acid) and the mixture boiled for 35 min. The solution was then cooled to room temperature and TBA expressed as absorbance units at 530 nm, measured using a 1 cm path length cuvette. A conversion factor of 6.2 (Crackel *et al.*, 1988) was used to convert absorbance unit to mg malondialdehyde (MDA)/kg whole milk powder.

3.4.6.6 Measurement of free sulphydryl groups

The amount of free sulphydryl groups in milk powder was measured using the procedure outlined by Kalab (1970). Ellman's reagent was prepared by dissolving 20 mg 5,5'-dithiobis-(2-nitrobenzoic acid) in 10 ml 0.1 M phoshate buffer, pH 7. 5 ml of the reconstituted milk powder (20 % (w/v)) was mixed with 0.1 ml Ellman's reagent, 1 ml of 0.2 M phosphate buffer, pH 8 and 4 ml distilled water. Cysteine was used as standard and absorbance at 412 nm was recorded. The concentration of free sulphydryl groups was expressed as μmoles/g powder.

3.4.6.7 Measurement of total sulphydryl groups

Total sulphydryl groups were measured using Ellman's reagent according to an adapted method of Beveridge *et al.* (1974). To 10 ml of a 20 % (w/v) solution of whole milk powder was added 1 ml 10 M urea in Tris-Glycine buffer (0.09 M Tris, 0.09 M glycine, pH 8.6 containing 4 mM EDTA) and 20 µl of 2-mercaptoethanol and the solution centrifuged at 5000 g for 10 min. The precipitate was washed twice with 5 ml 12 % (w/v) TCA and then dissolved in 3 ml 8 M urea in Tris-glycine buffer, pH 8.6 and 0.03 ml Ellman's reagent was added. Absorbance at 412 nm was recorded and total reactive sulphydryl groups expressed as µmoles/g powder.

3.4.6.8 Measurement of Vitamin E content

Vitamin E concentration of milk powder was determined using the method of Buttriss and Diplock (1984). To 1 ml of a 25 % (w/v) solution of whole milk powder was added 2 ml of a 1 % pyrogallol solution and 0.3 ml of a 50 % solution of KOH and the mixture saponified by shaking vigorously at 70° C for 30 min. The mixture was then allowed to cool on ice, after which 4 ml hexane containing 0.005 % butylated hydroxy toluene (BHT) and 1 ml water was added. The mixture was then centrifuged at 600 g for 10 min at 4° C. The upper layer was removed, dried under nitrogen and redissolved in 200 µl ethanol. Vitamin E was separated and quantified using a reverse phase HPLC with a nucleosil 100-5 C18 column (Macherey Nagel Art. 720014). The mobile phase was 97 % methanol and UV detection was at 292 nm. Results were expressed as µg/g powder.

3.4.6.9 Analysis of COPs

Lipids were extracted as described by Sander *et al.* (1989) and as outlined in Chapter 2, section 4.4. COPs were isolated from the twice hexane-washed lipid extract by solid phase extraction as described by Morgan and Armstrong (1989). COPs were then analysed by GC.FID using the method described in chapter 2, section 4.5. Results were expressed as µg/g lipid.

3.4.7 Statistical Methods

Whole milk powder was manufactured on 14 occasions. These occasions were grouped into three periods. The duration of each period was selected so that powders made during a specific period were manufactured from milk of similar composition,

and thus could be used as replicates in the statistical analysis. Periods 1, 2 and 3 had 5 (April 20-May 26; Trial number 1-5), 5 (June 16-July 21; Trial number 6-10) and 4 (August 3-October 21; Trial number 11-14) occasions as replicates, respectively. For each period COPs, WPNI, PVs and TBARS were analysed as a randomised block design with diet and heat treatment as a factorial combination. COPs at 12 months, PV and TBARS at 2, 4, 6 and 12 months were analysed as a split-plot design with diet and heat treatment combined factorially in the main plots and temperature and package type as the sub-plot factorial combination. Sulphydryl groups were analysed as a randomised block design with diet and heat treatment as the factors. Sulphydryl group analysis was done for a mid-lactation period with milk powder made on four separate occasions as replicates and for a late lactation period using thee separate occasions as replicates. Statistical analysis was completed using the GENSTAT package (The Numerical Algorithims Group Ltd., Oxford, UK).

3.5 RESULTS

3.5.1 Effect of animal feed quality on milkfat content

Animal feeding regimen had no significant effect on milkfat levels in the grass-fed herd (R) and grass plus concentrate-fed herd (S). Fig. 3.2 shows the fat levels in these milks, which ranged from 3.57 to 4.59 % for milk of herd R and from 3.77 to 5.05 % for the milk of herd S. An increase in milkfat levels was observed in milk of the grass plus concentrate-fed herd in late-lactation, though it was not statistically significant. Similarly, animal feeding regimen had no significant effect on fatty acid composition of milks obtained from these two herds. Table 3.2 shows the levels of fatty acids in milk from the grass-fed and grass plus concentrate-fed herds in early and mid-lactation. Milkfat content of short chain saturated fatty acids (C4-C12) were in general, much lower than the levels of longer chain saturated fatty acids (C14 - C18), the exception being the C20:0 saturated fatty acid (arachidic acid) content which was present in low concentrations (Table 3.1). Levels of even numbered saturated fatty acids (C4 - C16) were similar in milkfat from both herds. No significant difference was observed between total C18 fatty acids from the grass-fed herd (44.1 g FAME/100 g) and grass plus concentrate-fed herd (41.99 g FAME/100g).

3.5.2 Effect of animal feed quality on milk protein content

Fig. 3.3 (a) shows the percentage total protein in standardised milk of the grass-fed and grass plus concentrate-fed herds. Milk from the grass plus concentrate-fed herd (herd S) had significantly higher protein content ($p < 0.001$) than milk of the grass only-fed herd (herd R) thoughout the feeding study. Protein concentration was approximately 10 % lower in milk from herd R (2.96 to 3.66 %) than the herd S (3.34 to 4.02 %). Total protein levels increased in late-lactation (periods 11-14), though not significantly. Fig. 3.3 (b) shows the non-protein-nitrogen (NPN) in standardised milk of grass-fed and grass plus concentrate-fed herds. NPN content of milk from the 2 herds was not significantly different thoughout the study. Fig. 3.4 (a) shows data obtained for true protein content, defined as total protein less NPN in standardised milks from herds R and S. The pattern for true protein content followed

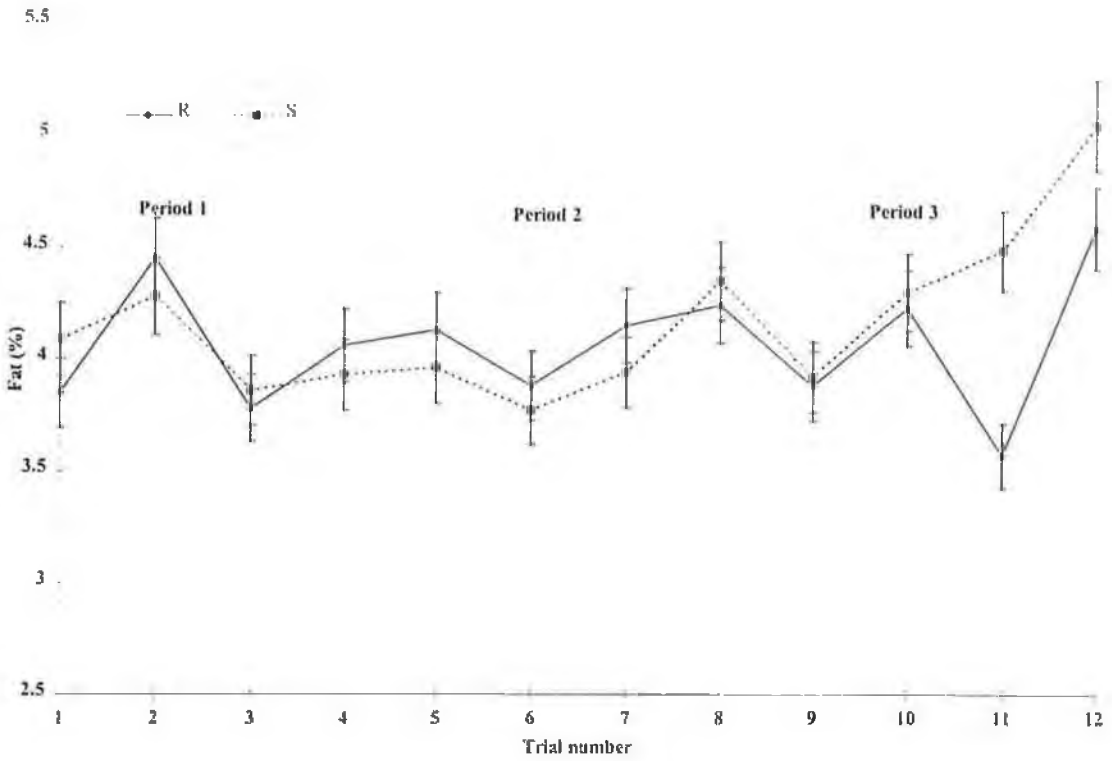


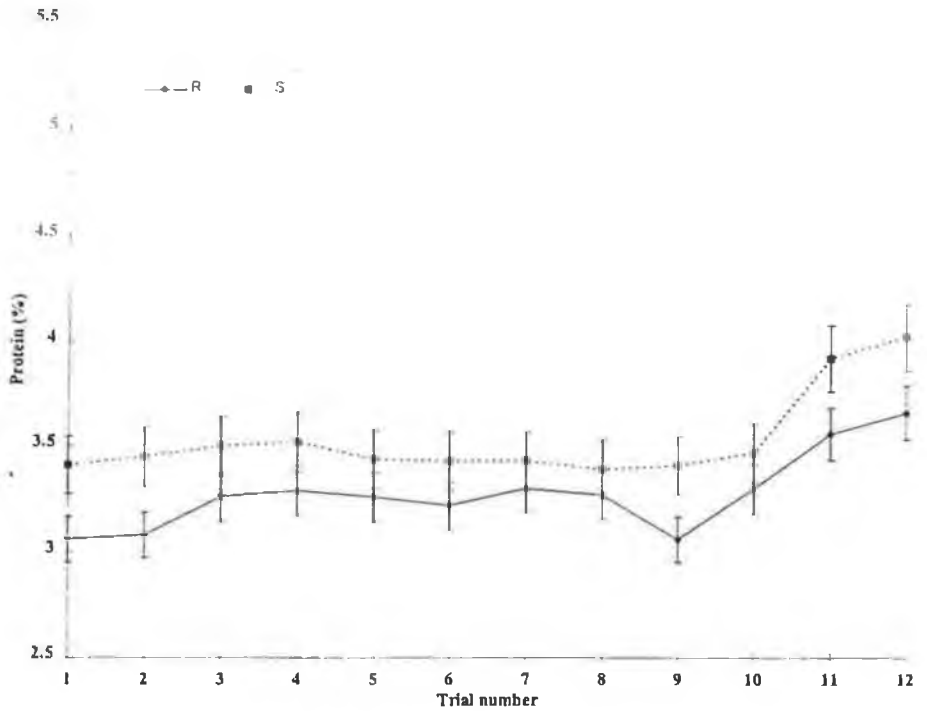
Fig. 3.2. Fat levels (%) in standardised milk of grass-fed herd (R) and grass plus concentrate-fed herd (S). Period 1 from April 20-May 26; Period 2 from June 16 to July 21 and Period 3 from August 3 to October 21. Error bars refer to mean ± standard deviation.

Table 3.1 Fatty acid composition (g FA as methylester/100 g of total FAME) of raw milks from the grass-fed herd (R) and grass plus concentrate-fed herd (S) during early and mid lactation.

Fatty acid	R[a]	S[a]
C4:0	3.34 ± 0.16	3.28 ± 0.12
C6:0	2.17 ± 0.13	2.20 ± 0.09
C8:0	1.28 ± 0.11	1.34 ± 0.08
C10:0	2.70 ± 0.33	2.98 ± 0.33
C12:	2.95 ± 0.38	3.32 ± 0.40
C14:0	9.66 ± 0.77	10.46 ± 0.51
C14:1	1.06 ± 0.12	1.08 ± 0.06
C16:0	23.17 ± 1.06	23.80 ± 0.68
C16:1	1.87 ± 0.12	1.73 ± 0.12
C18:0	12.82 ± 0.70	12.32 ± 0.76
C18:1	28.71 ± 1.82	26.94 ± 1.38
C18:2	1.68 ± 0.39	1.96 ± 0.13
C18:3	0.85 ± 0.11	0.77 ± 0.08
C20:0	0.16 ± 0.02	0.15 ± 0.04

[a] n= 9 periods

(a)



(b)

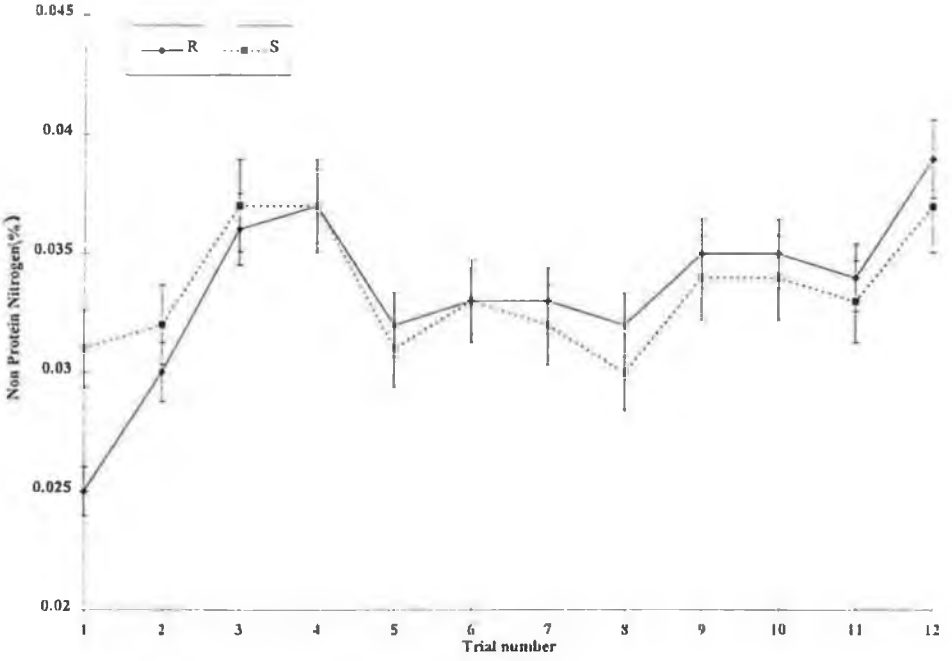
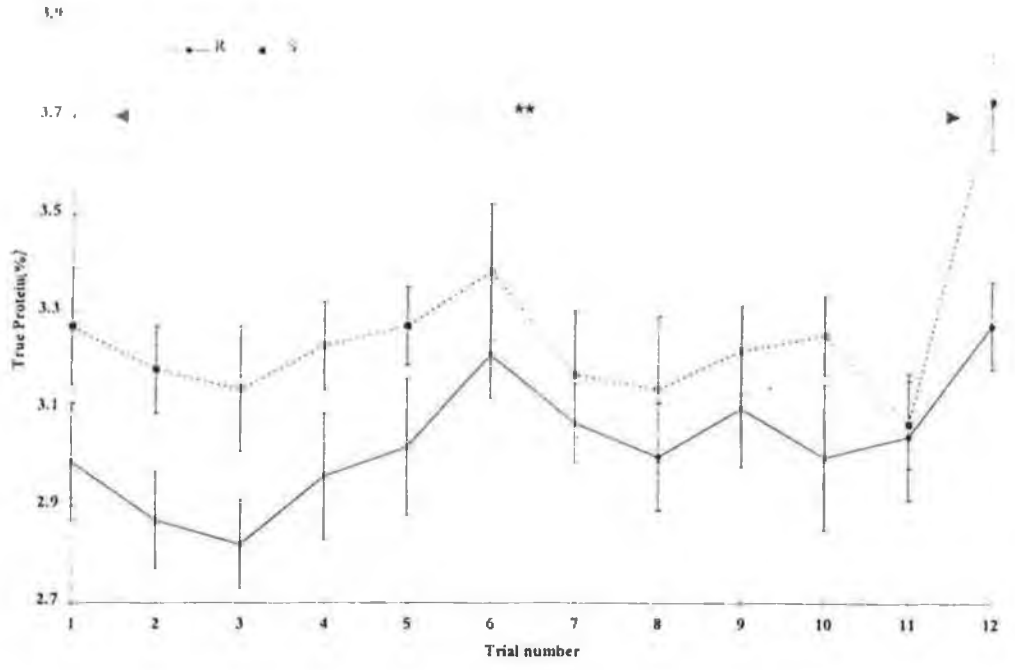


Fig. 3.3. (a) Total protein and (b) non-protein nitrogen of standardised milks from grass-fed herd (R) and grass plus concentrate-fed herd (S). Grass-fed significantly higher than grass plus concentrate-fed over full experimental period *** ($p < 0.001$). Error bars refer to mean ± standard deviation.

(a)



(b)

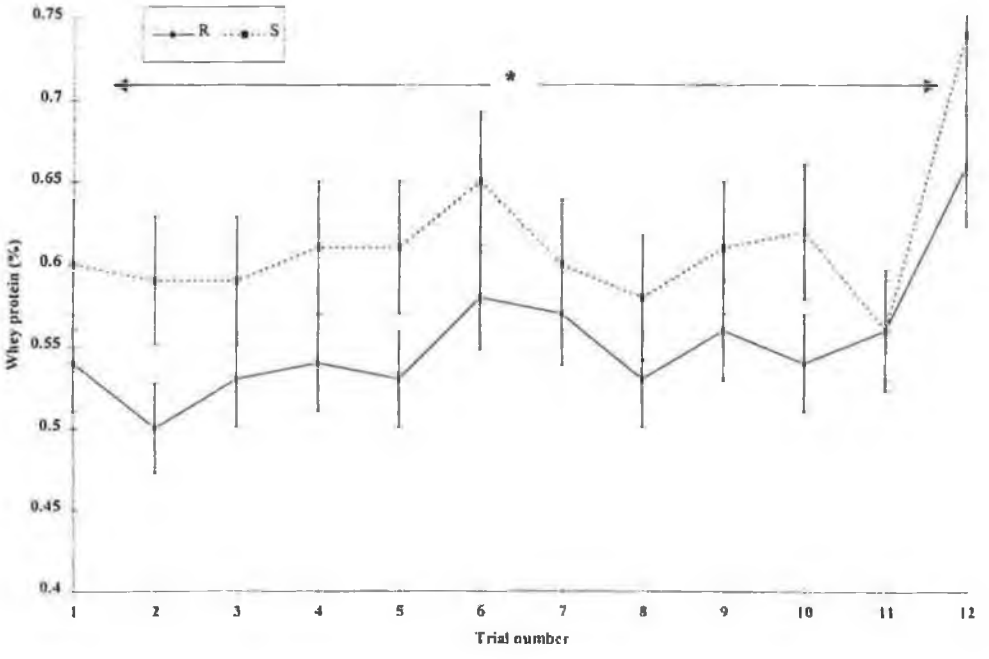


Fig. 3.4. (a) True protein and (b) whey protein of standardised milks from grass-fed herd (R) and grass plus concentrate herd (S). Grass-fed significantly higher than grass plus concentrate-fed over full experimental period ** ($p < 0.01$), * ($p < 0.05$). Error bars refer to mean ± standard deviation.

that of total protein, indicating that milk of herd R had significantly lower ($p < 0.01$) true-protein than that of the grass plus concentrate-fed herd. The level of true protein in milk from the grass plus concentrate-fed herd increased, though not significantly, in late-lactation. Whey protein was also significantly higher ($p < 0.05$) in milk of herd S than that of herd S (Fig. 3.4 (b)) and increased in late-lactation for both herds. Fig. 3.5 (a) shows the casein protein levels in milk from both herds. Casein levels followed a similar trend to that of protein content with milk of herd R showing significantly lower ($p < 0.001$) casein than milk of herd S. Similar to total protein and whey protein, casein increased markedly in the milk of herd S during late lactation. Animal feed quality had no significant effect on casein number (casein protein expressed as a percentage of total protein) over the full experimental period (Fig. 3.5 (b)). Table 3.2 summarises the effect of animal feed regimen and stage of lactation on protein composition (Figs. 3.3 - 3.5).

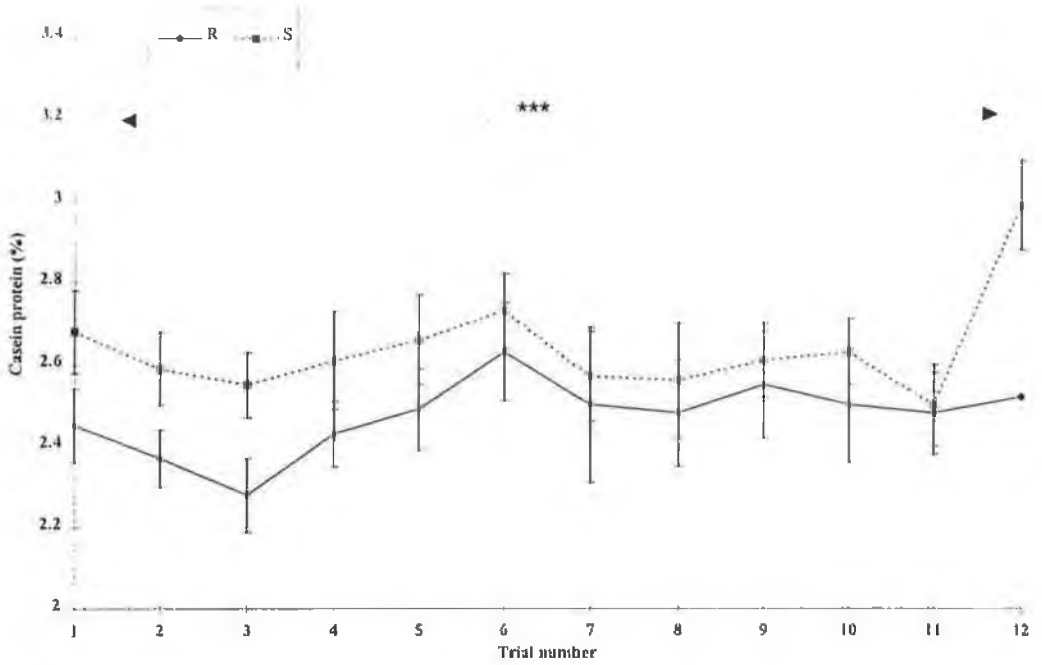
3.5.3 Effect of animal feed regimen on undenatured whey protein nitrogen index of whole milk powder

Whole milk powder was produced from milk of grass-fed and grass plus concentrate-fed herds following low pre-heat treatment. The effects of animal feeding regimen on whey protein nitrogen index (WPNI) values of the whole milk powders were compared. The WPNI values obtained for the low-heat powders of herds R and S were 6.9 ± 0.9 and 7.8 ± 1.1 mg/g powder, respectively. Animal feeding regimen significantly ($p < 0.05$) affected levels of undenatured whey protein in whole milk powders only during period 1, but had no effect on powders produced in periods 2 and 3 (Fig. 3.6).

3.5.4 Effect of animal feeding regimen on PV levels in whole milk powder

The primary oxidative stability of fresh and stored low-heat whole milk powders was assessed by measuring peroxide values (PV). Fig. 3.7 (a) shows the PV (mEq O_2/kg fat) levels obtained in fresh milk powders manufactured thoughout the experimental period.

(a)



(b)

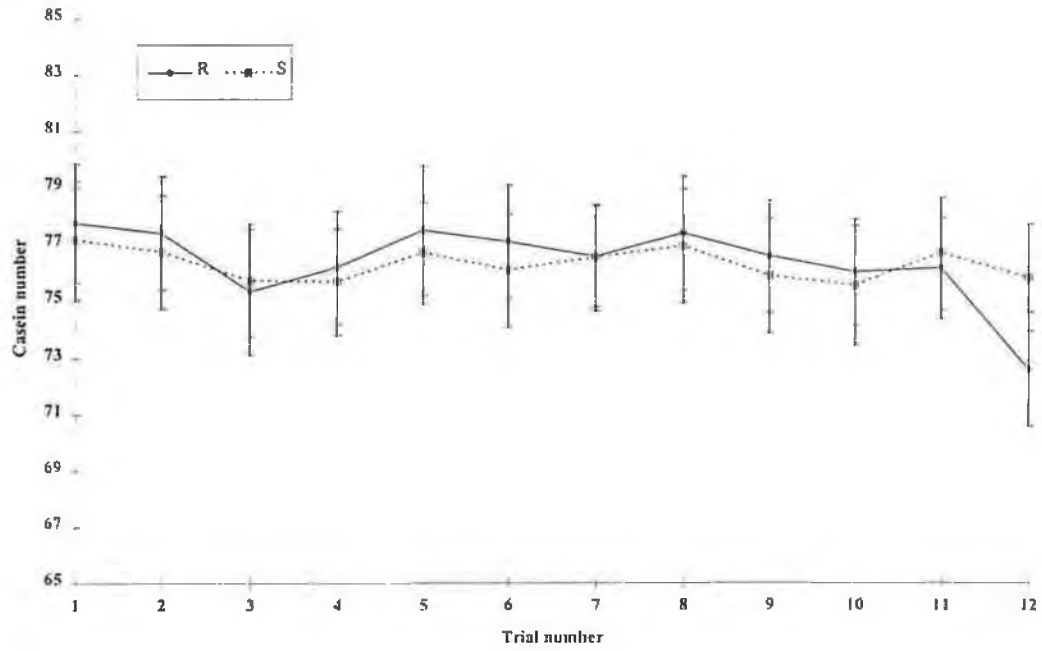


Fig. 3.5. (a) Casein protein and (b) casein number of standardised milks from grass-fed herd (R) and grass plus concentrate-fed herd (S). Grass-fed significantly higher than grass plus concentrate-fed over full experimental period *** ($p < 0.001$). Error bars refer to mean ± standard deviation.

Table 3.2 Effect of animal feed quality and period of milking on protein fractions in milk. Values refer to probability levels.

Effect	Total Protein	True Protein	Casein Protein	Casein Number	Non Protein Nitrogen	Whey Protein
Animal Feeding Regimen	0.001	0.01	0.001	N.S.	N.S.	0.05
Stage of lactation	N.S.*	N.S.	N.S.	N.S.	N.S.	N.S.

* N.S. : not statistically significant.

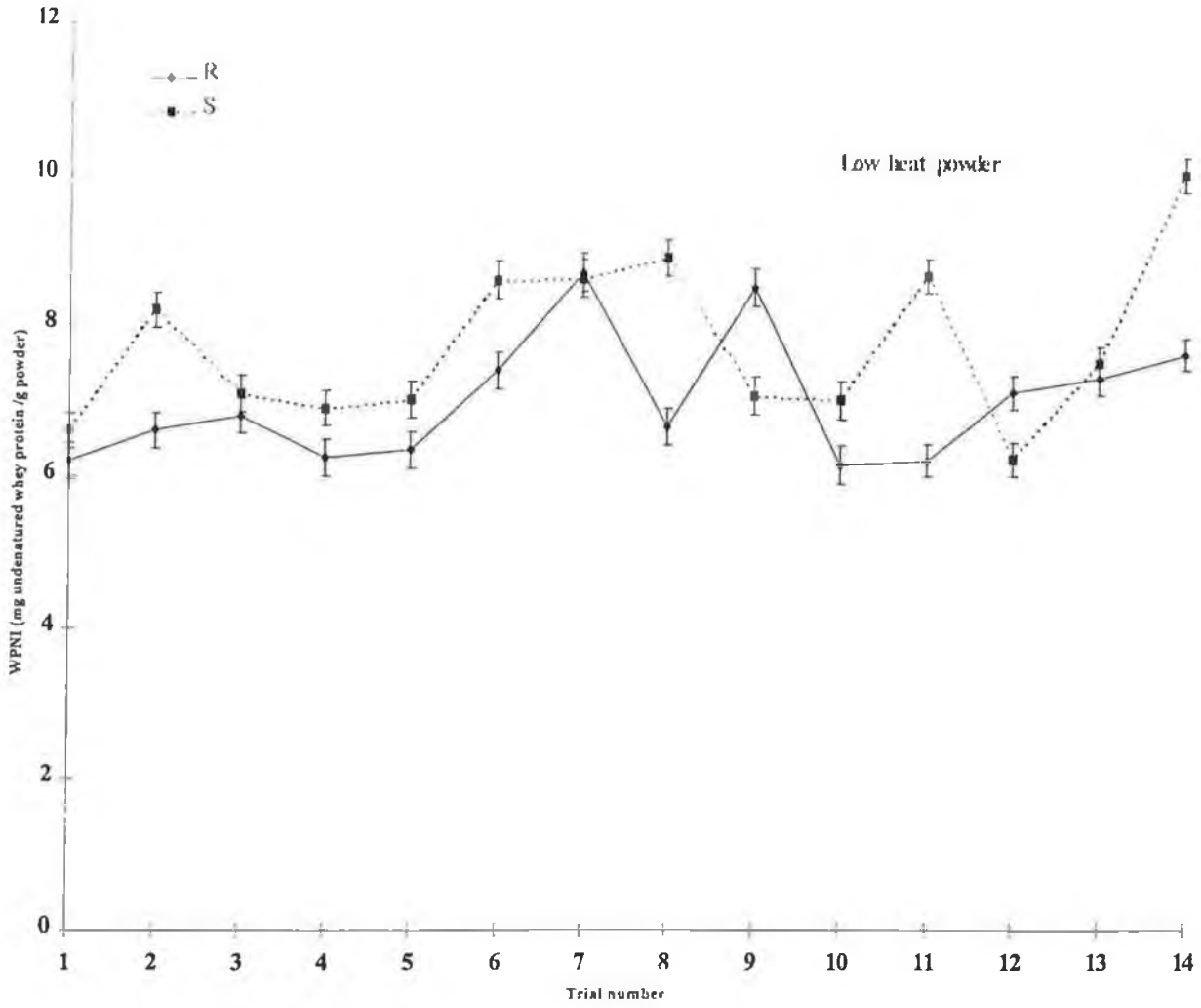


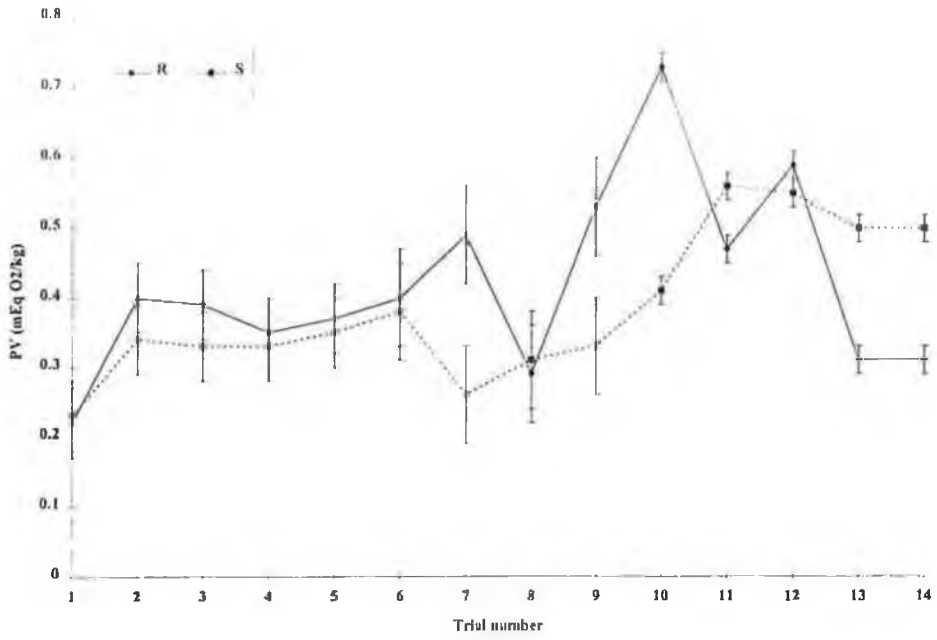
Fig. 3.6 Undenatured whey protein nitrogen index (WPNI) of low-heat powders from grass-fed herd (R) and grass plus concentrate-fed herd (S). Grass-fed significantly higher than grass plus concentrate-fed * ($p < 0.05$). Error bars refer to mean ± standard deviation.

PV levels of powders manufactured during periods 1 and 2 were significantly higher ($p < 0.05$) for the grass-fed herd than the grass plus concentrate-fed herd,while in PV levels of powders manufactured during periods 1 and 2 were significantly higher ($p < 0.05$) for the grass-fed herd than the grass plus concentrate-fed herd, while in PV levels of powders manufactured during periods 1 and 2 were significantly higher ($p < 0.05$) for the grass-fed herd than the grass plus concentrate-fed herd, while in fed herd showed higher PV levels (up to 0.5 mEq O_2/kg) than the grass-fed herd herd (0.31 mEq O_2/kg.). Fig. 3.7 (b) shows the changes in PV values obtained when powders were sachet-packed and stored for up to 12 months at 15° C. PV values increased in both powders reaching a maximum after 6 months storage at 15° C and declined thereafter. Maximum PV reached was 2.9 mEq O_2/kg for powder of the grass-fed herd and 2.7 mEq O_2/kg for powders of herd S after 6 months of storage. In general, whole milk powders from the grass plus concentrate-fed herd that were sachet-packed and stored at 15° C had lower levels of lipid oxidation as indicated by lower PV compared with whole milk powder from the grass-fed herd. Concentrate feeding had a significant inhibitory ($p < 0.05$) effect on peroxide development in whole milk powder after 12 months storage at 15° C.

3.5.5 Effect of animal feeding regimen on TBARS in whole milk powder

Fig. 3.8 (a) shows the levels of TBARS (mg MDA/kg) obtained in fresh milk powders manufactured thoughout the experimental period. Animal feeding regimen had a significant ($p < 0.05$) effect on TBARS in fresh whole milk powders in periods 1 and 2 only, though the effect was not in the same direction as for PV. As seen for PV levels, TBARS increased in powders from both herds when stored in foil-lined paper bags at 15° C for up to 12 months (Fig. 3.8 b). TBARS continued to increase steadily thoughout the entire 12 month storage period, increasing more rapidly in the second half of the storage study, unlike PV levels which increased only during the first 6 months of the storage study (Fig. 3.7 b). This inverse relationship between PV and TBARS after 6 months storage may indicate progression of oxidation from a primary to a secondary state. TBARS in whole milk powder of the grass-fed herd

(a)



(b)

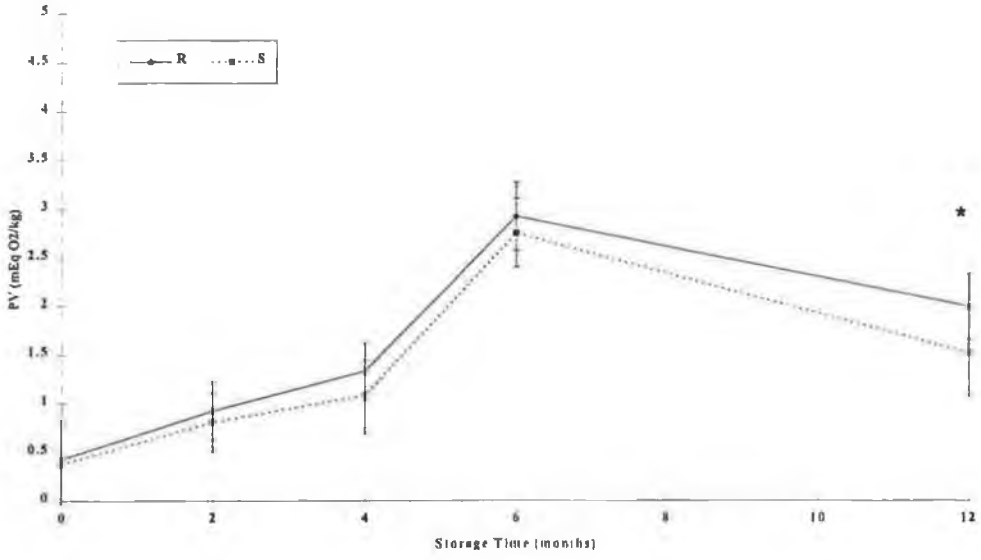
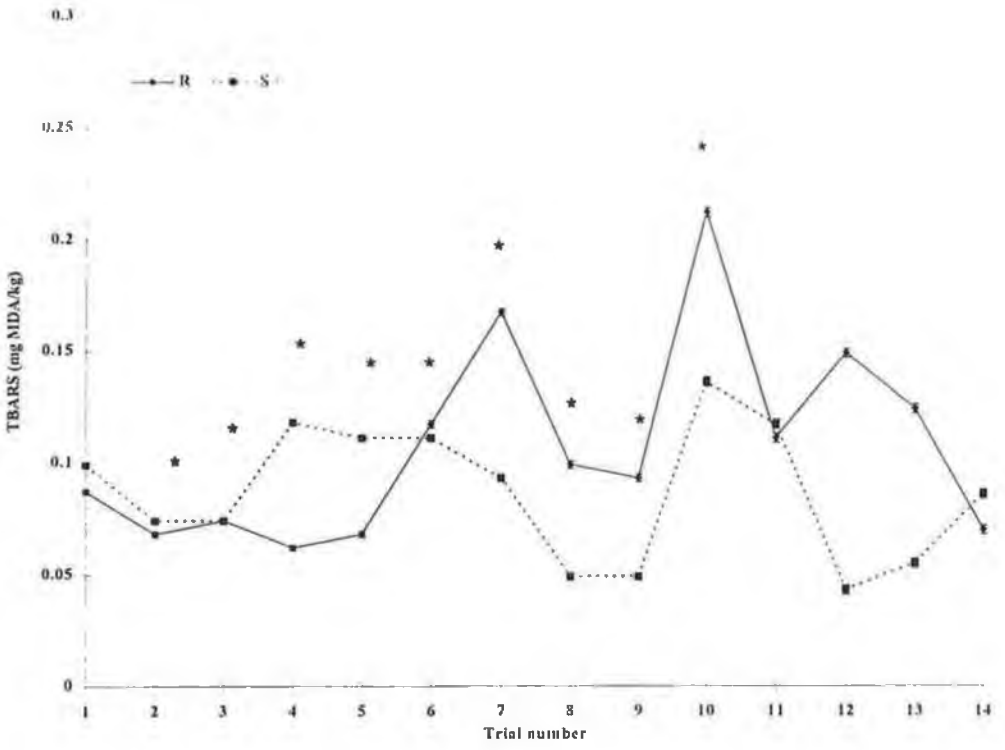


Fig. 3.7. PV levels in (a) fresh and (b) sachet-packed whole milk powders stored at 15° C from grass-fed herd (R) and grass plus concentrate-fed herd (S). Grass-fed significantly higher than grass plus concentrate-fed * ($p < 0.05$). Error bars refer to mean ± standard deviation.

(a)



(b)

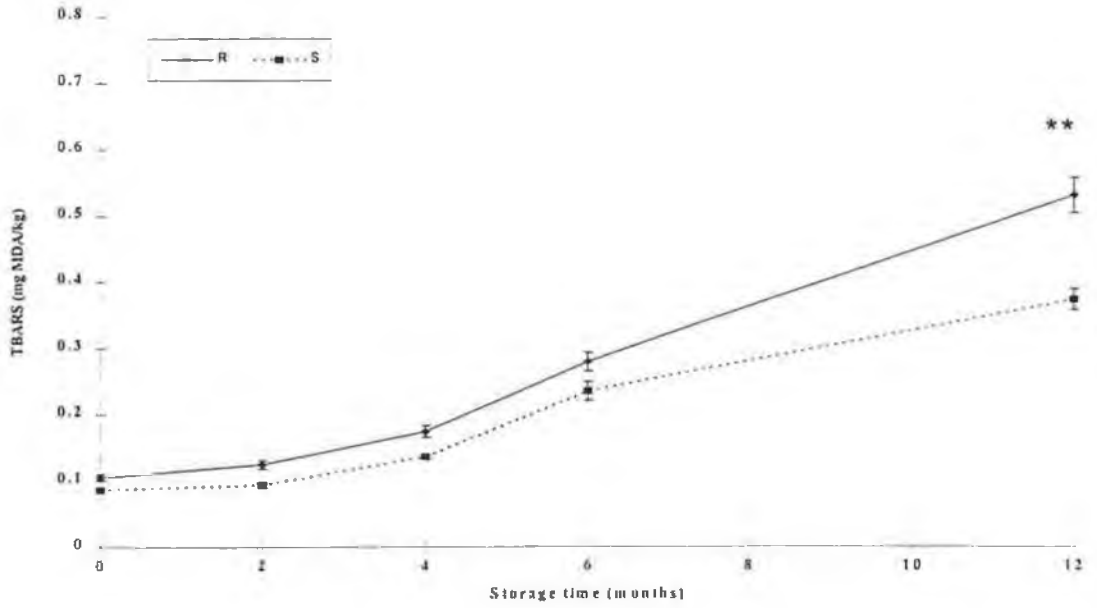


Fig. 3.8. TBARS levels in (a) fresh whole milk powder and (b) sachet-packed whole milk powders stored at 15° C from grass-fed herd (R) and grass plus concentrate-fed (S) herd. Grass-fed significantly higher than grass plus concentrate-fed * ($p < 0.05$) ** ($p < 0.01$) was significantly higher ($p < 0.01$) than whole milk powder of the concentrate-fed herd after 12 months. Error bars refer to mean ± standard deviation.

3.5.6 Effect of animal feed quality on cholesterol oxidation in whole milk powder

The data obtained for total cholesterol oxidation products (COPs) expressed as μg/g lipid for fresh whole milk powders manufactured from both grass and grass plus concentrate-fed herds are shown in Fig. 3.9. Total COPs levels remained below 1 μg/g for the duration of the study and were not significantly different in powders manufactured with milk of the grass-fed herd compared with the grass plus concentrate-fed herd. Storage of whole milk powder from both herds yielded significantly higher ($p < 0.001$) levels of COPs relative to fresh whole milk powder and Table 3.3 shows the levels of individual and total COPs (expressed as μg/g and as a % total cholesterol) when these powders were sachet-packed and stored for 12 months at 15° C. Following storage, 7-ketocholesterol was the predominant cholesterol oxide detected in milk powders of both herds. 7-Ketocholesterol and cholesterol-β-epoxide were significantly higher ($p < 0.05$) in whole milk powder of the grass-fed herd compared with the grass plus concentrate-fed herd. Neither 25-hydroxycholesterol or cholestanetriol were detected in whole milk powders of both herds, which were sachet-packed and stored at 15° C for 12 months. The level of total COPs in stored powders of the grass-fed (5.50 ± 1.27 μg/g) herd was approximately 5-fold higher relative to fresh milk powders of the same herd. This represented 0.27 % of total cholesterol (2000 μg/g fat) in milk being oxidised during the storage period. The level of total COPs observed in stored powders of the grass plus concentrate-fed herd (3.89 ± 1.01 μg/g) was significantly lower ($p < 0.05$) than powders of the grass-fed herd, and amounted to 0.19 % of total cholesterol being oxidised during the 12 months storage period (Table 3.3).

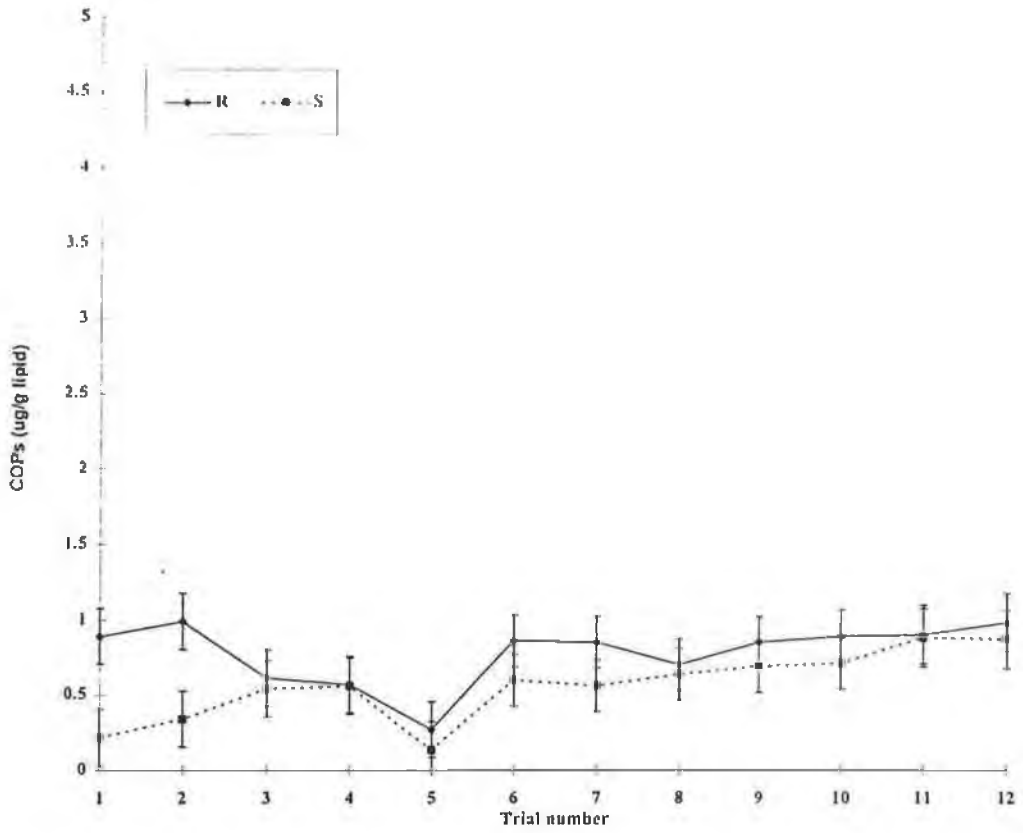


Fig.3.9. COPs levels in fresh low-heat whole milk powders manufactured from milks of grass-fed (R) and grass plus concentrate-fed (S) herds. Error bars refer to mean ± standard deviation.

Table 3.3 Levels of COPs in 12 month old sachet-packed whole milk powders after storage at 15°C. Levels of primary and secondary oxidation products are shown as well as the total level of COPs expressed as μg/g and as % total cholesterol.

Whole milk powder	7-Keto	25-OH	α-epoxide	β-epoxide	Triol	Total (μg/g lipid)	Total (% total cholesterol)
R	4.18 ± 0.94 *	-	0.65 ± 0.11	0.67 ± 0.9 *	-	5.5 ± 1.27 *	0.27 ± 0.063 *
S	3.42 ± 0.89	-	0.47 ± 0.08	-	-	3.89 ± 1.01	0.19 ± 0.05

* Grass-fed herd (R) ($p < 0.05$) significantly higher than grass plus concentrate-fed herd (S).

3.5.6 Effect of animal feed quality on sulphydryl groups in whole milk powder

Table 3.4 demonstrates the levels of free and total sulphydryl groups found in stored powders thoughout this study. Animal feed quality had a significant effect ($p < 0.05$) on free sulphydryl levels, with whole milk powder from the grass plus concentrate-fed herd containing higher levels of free sulphydryl groups (0.11 ± 0.04 μmoles/g powder) than whole milk powder from the grass-fed herd (0.07 ± 0.02 μmoles/g powder). Whole milk powders from the grass plus concentrate-fed herd also had higher levels of total sulphydryl groups (16.88 ± 0.03 μmoles/g powder) compared with those from the grass-fed herd (15.91 ± 0.03 μmoles/g powder).

Table 3.4 Free sulphydryl and total sulphydryl group levels in whole milk powder of grass-fed herd (R) and grass plus concentrate-fed herd (S).

Herd	**Free Sulphydryl μmoles/g powder** [a]	**Total Sulphydryl μmoles/g powder** [a]
R	0.07 ± 0.02	15.91 ± 0.03
S	0.11 ± 0.04 *	16.88 ± 0.03

[a] n (number of samples analysed)=7

* Grass plus concentrate-fed herd significantly higher ($p < 0.05$) than grass-fed herd.

3.6 DISCUSSION

Milk from both herds R and S was analysed for fat and protein contents prior to low-heat powder manufacture. The oxidative stability of these low-heat powders was assessed during 'normal' commercial storage conditions (i.e. sachet-packed and stored at 15° C). In this way, the influence of animal feeding regimen on milk quality could be addressed. It should be emphasised at this stage however that only the relative effects of dietary restriction and supplementation could be evaluated, as a control herd fed on the standard grassland and stocking management system was not included in the study. The grass only-fed herd differed from the standard grassland and stocking management system in having a lower land allocation, a lower stocking rate and no concentrate supplementation.

Effect of animal feeding regimen on milkfat content

The results presented in this chapter report the effects of grass feed only compared to grass plus concentrate feed on oxidative stability of whole milk powder in addition to the influence of stage of lactation on fat and protein levels prior to whole milk powder manufacture. The milkfat levels analysed in the present study ranged from 3.78 - 5.05 % and the data suggests that the 2 dietary treatments did not significantly influence milkfat content. Previous reports of fat levels in milk range from 2.14 to 4.49 % (Sutton, 1989) (Fig. 3.2). Variation in fat levels can be attributed to a number of factors including stage of lactation, dietary intake, breed, health of animal and climate (Cullinane *et al.*, 1984) Though not significant, an increase was observed in milkfat of the grass plus concentrate-fed herd in period 3, coinciding with late lactation. Milkfat increases in late-lactation have been previously reported (Walstra and Jenness 1984; Early, 1992). Walstra and Jenness (1984) reported that the time elapsed after parturition (calving) considerably influenced milk composition, though the extent to which milk composition changed with lactation varied markedly among animals. It is difficult to establish the precise effect of lactation stage as both season and feeding regimens change with advancing lactation.

In the present study, milkfat levels increased slighty, though not significantly, in the grass plus concentrate-fed animals but not in the grass-fed herd, suggesting that milkfat content during late-lactation was influenced by animal feeding regimen, coinciding with the earlier study of McDowall (1962). Hence the absence of a late-lactation effect on fat content that was observed in the grass-fed herd may be accounted for by the lower plane of nutrition.

Similarly, the two dietary regimens investigated had no significant effect on fatty acid composition of milk, though there was a small trend towards increased levels of total C18 unsaturated fatty acids in the grass-fed herd (Table 3.1). A state of negative energy balance during early to mid lactation has been reported to result in mobilisation of adipose tissue reserves (Parodi, 1974; Palmquist *et al.*, 1993), which have a high content of long chain fatty acids and may account for the slightly higher level of total C18 fatty acids in the milk from herd R up to and including mid-lactation. However, the uptake of long chain fatty acids was not sufficiently high to inhibit *de novo* synthesis of short chain fatty acids by the mammary tissue since similar levels of these short chain fatty acids were observed in milk from both herds. Luick and Smith (1963) reported a decrease in the proportions of fatty acids of chain length C4 to C16 and an increase in C18:0 and C18:1 as a result of the mobilisation of body reserves when cows are placed on a 'restricted' intake.

Effect of animal feeding regimen on milk protein content

Protein levels ranged from 2.96 to 4.02 % in the present study and concentrate feeding had a significant effect ($p < 0.001$) on milk protein content (Fig. 3.3 a). Previous studies have also shown that feeding higher levels of concentrate supplement, thereby increasing the plane of animal nutrition increases both milk protein concentration and yield (Rook, 1992; O' Mara, 1993). In the present study, milk obtained from the grass-fed herd had a total protein content of less than 3.3 % between April and October. Though not significant, the total protein content increased from September to October, which may be attributable to a late-lactation effect. It has previously been reported that protein levels vary according to stage of

lactation (Rook, 1961; Rook, 1971; Faegan, 1979; De Peters and Cant, 1992; Murphy and O'Mara, 1993). Rook (1971) reported that in days immediately before calving, milk secretion accumulates within the udder and during that time there is considerable resorption of lactose, but a negligible resorption of protein and hence the high level of protein in early lactation milk. The relationship between protein and lactose synthesis is due to the role of α-lactalbumin in lactose synthesis in the mammary gland (Morrissey, 1985). The secretion of α-lactalbumin controls the levels of of lactose synthesis and a high correlation has been found between lactose and α-lactalbumin in milk (Morrissey, 1985).

The pattern for true protein content followed that of total protein, where again, the grass-fed herd had significantly lower ($p < 0.01$) levels (Fig. 3.4 a). The casein levels followed similar trends to that of total protein in the present study (Fig. 3.5). In late-lactation milk, the casein content increased as total protein increased. This observation was reported also by De Peters and Cant (1992). In the present study, the casein number was similar for both herds thoughout lactation, showing that the ratio of casein to total protein was maintained, although the total protein was different at the two nutritional levels. The casein number declined slightly from September onwards for both herds. This may be due to the degradation of β-casein in the mammary gland by the milk enzyme plasmin, which is a phenomenon of late-lactation milk (Oltner *et al.*, 1983).

The grass only diet had a negative impact on the whey protein content of milk, with milk of the grass-fed herd containing significantly lower ($p < 0.05$) levels than that of the grass plus concentrate-fed herd Fig. 3.4 b). This could have serious implications in the manufacture of whole milk powder from milk of grass-fed herd, as milk powder is classified on the basis of native whey protein in the powder after heat treatment. High-heat powder is classified as containing less than or equal to 1.5 mg whey protein/g powder, whereas low-heat is classified as containing greater than or equal to 6.0 mg whey protein/g powder (ADMI, 1971). Therefore, if the concentration of whey protein is low in the raw milk, medium-heat powder

subsequently manufactured could be incorrectly classified as high-heat powder, leading to problems in the production of consistent quality powder.

A recent study on the influence of diet on Cheddar cheese manufacture (Kefford *et al.*, 1995) found that the plane of nutrition also had a significant increase ($p < 0.05$) on casein and whey protein, as seen in the present study, and no significant effect on milkfat levels. However, direct comparisons cannot be made, as the concentrate used by Kefford *et al.* (1995) was different to that of the present study in that it contained only maize grain (as energy source) and sunflower meal (as protein source).

Effect of animal feeding regimen on undenatured whey protein nitrogen index of whole milk powder

Animal feeding regimen had no overall effect on WPNI of low-heat powder of both the grass-fed herd R and the grass plus concentrate-fed herd S although whole milk powder of herd S had significantly higher WPNI values than that of herd S in period 1 ($p < 0.05$). Though not significant over the entire experimental period, the WPNI of powders produced from the grass plus concentrate-fed herd showed a trend towards an increase, which may be accounted for by the significantly higher ($p < 0.05$) whey protein content (Fig. 3.6).

Effect of animal feeding regimen on oxidative stability of whole milk powder

While animal feeding regimen had no overall significant effect on PV or TBARS in fresh whole milk powder, fresh milk powder from the grass-fed herd did show significantly elevated ($p < 0.05$) levels of PV and TBARS in periods 1 and 2. On the other hand, whole milk powders from the grass plus concentrate-fed herd, when sachet-packed and stored at 15° C for 12 months, had lower levels of lipid oxidation as indicated by lower PV and TBARS values compared with whole milk powder from the grass-fed herd.

When whole milk powders were sachet-packed and stored at 15° C for 12 months, total COPs levels were significantly lower ($p < 0.05$) for powders of the grass plus concentrate-fed herd compared with those of the herd fed grass only (Fig. 3.9). 25-hydroxycholesterol and cholestanetriol were not detected and 7-ketocholesterol and cholesterol-β-epoxide were significantly lower ($p < 0.05$) in whole milk powder of the grass plus concentrate-fed herd than that of the grass-fed herd. Though not significant, the grass-fed herd showed slightly higher levels of oleic, linoleic and linolenic acids and it has been reported that cholesterol in foods is oxidised when it exists together with unsaturated fats (Osada *et al.*, 1993). This may have been a contributory factor to the increased presence of cholesterol oxides in powders of the grass-fed herd.

The data from the present study indicated differences in cholesterol oxides in stored milk powders from both grass-fed and grass plus concentrate-fed herds, with the whole milk powder of the grass-fed herd showing higher levels following 12 months storage (Table 3.4). 7-Ketocholesterol was found to be the predominant cholesterol oxide present, followed by the epimeric epoxides, coinciding with findings of Chan *et al.* (1993). 7-Ketocholesterol, a primary oxidation product, has been reported as a principal cholesterol oxide (Maerker, 1987) and has been used as a marker of cholesterol oxidation in ground beef (De Vore, 1988). Cholestanetriol, a secondary oxidation product, is a degradation product of 7-ketocholesterol and is only found in extremely oxidised powders. The stored powders in the present study contained total COPs levels of less than 6 μg/g, which is low relative to other foodstuffs. Dried egg powders have been shown to contain up to 300 μg/g total COPs (Nourooz-Zadeh and Appelqvist, 1987; Van de Bovenkamp *et al.*, 1988).

The protective effect against oxidation offered by the milk of the grass plus concentrate-fed herd would indicate that supplementary concentrate feeding was beneficial. A number of factors need to be considered in order to account for this effect. It has been previously reported that animals supplemented with vitamin E showed higher vitamin E content in milk compared with grazing animals (Atwal *et al.*, 1991). The vitamin E concentration of the concentrate supplement fed to the

grass plus concentrate fed herd in this study was 22.5 IU vitamin E/ day. Analysis of vitamin E in whole milk powders from both herds did not show a significant difference, although the levels were slightly higher (14 ± 0.8 μg/g), in the grass plus concentrate fed-herd compared to the grass-fed herd (11 ± 0.5 μg/g). Herd R was fed on grass only, and the vitamin E in the milk powder would therefore have been furnished by grass intake, which has been reported to range from 9 μg/g (September) to 350 μg/g (April) Brown (1953). Bovine milk typically contains an average of 20-50 μg/g vitamin E (McGillivay, 1956; O'Shea, personal communication). The vitamin E level of 14 ± 0.8 μg/g in the milk powders of the grass plus concentrate-fed herd may have been a result of the extra vitamin E intake, as a result of concentrate feeding. The levels observed in these powders were consistent with those found in commercial powders (O'Shea, 1996 personal communication). This may have been due to losses incurred on processing and it has been reported (Dziezak, 1986) that vitamin E can be lost during refining and processing conditions. However, it is more likely that the similar vitamin E levels found may be due to the fact that the levels in the concentrate were too low to have any significant effect (Murphy 1996, personal communication). In the present study, the vitamin E levels in the concentrate were very low (22.5 IU/day) compared with other studies where levels ranging from 400 IU/day to 1 g/day (1 mg = 1.49 IU) were incorporated (Atwal *et al.*, 1990; Ashes *et al.*, 1992; Wahle *et al.*, 1993). In addition, the transfer of vitamin E from feed has been reported to be as low as 2 % (King, 1968), hence the need for incorporation of very high levels in feed to bring about a significant increase in the resultant milk. It is worth reiterating that the aim of the study was to investigate the protective effect of concentrate feeding, rather than investigating dose responses of α-tocopherol.

The increased intakes of antioxidant minerals and vitamins in the concentrate, in addition to vitamin E may be expected to contribute to the increased oxidative stability offered by the milk of the concentrate-fed herd. Among the minerals and vitamins added, manganese, copper and zinc are essential in their functioning in the active site of free radical scavenging enzymes such as the superoxide dismutases (Mn SOD and Cu Zn SOD) (Halliwell, 1994). A possible increased activity of

SOD in the milk of the grass plus concentrate-fed herd may have accounted for the increased oxidative stability seen in whole milk powders manufactured, although this was not measured in the present study.

Tallow was incorporated as the lipid source in the concentrate feed. Effects of dietary fat on milkfat levels and composition have been studied more than any other factor (Claperton and Banks, 1985; Grummer, 1991, Palmquist *et al.*, 1993). The effects of dietary lipids on milkfat synthesis are complex (Sutton, 1989) and are mediated within the rumen, body and udder of the cow (Storry, 1981). In the present study, however, supplemental lipids had no significant effect on the fatty acid profiles, though slightly higher levels of unsaturated fatty acids were observed in milk of the grass-fed herd.

Effects of dietary protein on milkfat composition are less well documented (Palmquist *et al.*, 1993). The protein sources of the concentrate-fed to the grass plus concentrate-fed herd were rapeseed meal and corn gluten. High intakes of protein have been reported to result in mobilisation of adipose tissue in early lactation, which could increase the proportion of long chain fatty acids in milk. Protein intake may also have subtle effects on milk fatty acids by providing precursors for synthesis of various branched-chain fatty acids though ruminal degradation of dietary protein (Oldham, 1984). Again, the protein in the concentrate did not have a significant effect on the proportion of long chain fatty acids. However, a protein effect wasn't specifically examined.

Previous reports suggest that a grass only-fed diet can result in the production of increased levels of unsaturated C18 fatty acids, which can result in increased autoxidation (Sidhu *et al.*, 1976; Sutton, 1989). In the present study, however, the cows fed the grass only diet showed no significant differences in levels of oleic, linoleic and linolenic acids compared to cows fed grass plus concentrate. A possible explanation for the increased oxidation in the whole milk powders of the grass-fed herd may have been induction of lipolysis as Astrup *et al.* (1980) found that restricted feed intake induced lipolysis. However, the data presented in the present

study did not indicate any evidence of increased lipase activity in milk of the grass-fed herd as the levels of short chain fatty acids (C4 - C10) were similar in milk obtained from both herds.

Since concentrate feeding had no significant effect on milkfat level or composition in the present study, it is likely that the increased protein levels in the milk of the grass plus concentrate-fed herd may have been responsible for the protective effect observed in milk powder manufactured from this milk. Both the elevated whey and casein fractions observed have been previously reported to have antioxidant activity (Taylor and Richardson, 1980; Allen and Wrieden., 1982). Free sulphydryl groups are known to result in increased oxidative stability of whole milk powder by scavenging of free radicals (Beveridge *et al.*, 1974; Taylor and Richardson, 1980; Van Mil and Jans, 1991). Whole milk powders from the grass plus concentrate-fed herd had higher levels of reactive sulphydryl groups compared with those from the grass-fed herd (Table 3.4). This may be attributed to the increased whey protein levels in milk of the grass plus concentrate-fed herd relative to that of the grass-fed herd. However, Taylor and Richardson (1980) have demonstrated that sulphydryl groups are responsible for only part of the antioxidant activity. They found that β-lactoglobulin, which contains most of the sulphydryl groups of the milk (0.9 moles SH/mole protein (Fernandez-Diez *et al.*, 1964)), accounted for only a small portion of the total antioxidant activity, whereas the caseins, which have few or no sulphydryl groups (Beveridge and Nakai, 1970), had significant antioxidant effect when heated. In support of this finding, the casein content of the milk of the grass plus concentrate-fed herd was greater than that of the herd which received a grass only diet. Caseins possess significant antioxidant activity which may be related to their hydrophobic nature (Taylor and Richardson, 1980) and orientation of potential antioxidant side-chains of constituent amino acids at the lipid interface. Brunner (1974) reported a retardation of lipid oxidation in homogenized milk when milkfat droplets were resurfaced with casein. The antioxidant activity associated with casein almost certainly derives from it's ability to bind metals to phosphoseryl residues and the binding of metals to phosphate appears generally to inhibit their catalytic effect on autoxidation (Allen and Wrieden, 1982).

3.7 CONCLUSIONS

Increased dietary intake (i.e. concentrate-feeding and increased stocking rates) offered protection against oxidation of whole milk powder during storage expressed by reduced levels of lipid peroxides, secondary oxidation products and cholesterol oxidation products. Such protection may have been attributed to the total antioxidant activity of the increased levels of casein and whey proteins of milk of the grass plus concentrate-fed herd (although other factors may have also contributed). In addition, protection may have been offered by the increased minerals and vitamins in the concentrate feed thereby further contributing to the antioxidant intake of the grass plus concentrate-fed herd. Animal feeding regimen offers considerable potential for modifying milk composition and much progress has been made since Powell's original observations that diet had a significant effect on milkfat levels (Powell, 1938), but many areas of uncertainty remain. Research into nutritional manipulation of milk is likely to be limited until the Dairy industry recognises the potential to make significant progress in improving milk quality though feeding practices, and until technologies are developed and implemented which provide incentive for producers to improve milk quality (Grummer, 1991).

CHAPTER 4

EFFECT OF PROCESSING, PACKING AND STORAGE ON OXIDATIVE STABILITY IN WHOLE MILK POWDER

4.1 INTRODUCTION

Whole milk powder production in Ireland today amounts to 3600 % of our national requirements and constitutes the bulk of the Irish milk powder export market. A major part of this export is marketed in countries with a hot and often humid climate. This requires that whole milk powder have good shelf life (Ipsen and Hansen, 1988), which can be defined as the ability to retain a fresh and neutral flavour under given storage conditions. The storage stability of a powder also includes its ability to be reconstituted (Ipsen and Hansen, 1988).

The manufacture of spray-dried milk products includes a number of steps in addition to evaporation and spray-drying (Caric, 1994). These include pre-heat treatments, homogenisation, selection and modification of the spray-drying conditions, such as inlet and outlet temperatures. By incorporating a number of additional steps such as lecithinisation, it is possible to influence the product properties to a great extent, so as to produce specialised products (Pisecky, 1980).

Research endeavours on whole milk powder can be broadly classed under two headings: (i) the effect of age on the development of 'off-flavours' in milk powder and (ii) the effect of storage conditions on the solubility of whole milk powders (Tuohy, 1987; Nourooz-Zadeh and Appelqvist, 1988; Baldwin and Ackland, 1991). There is, however, a relative dearth of information on the effect of heat processing treatments during spray-drying on oxidative stability of whole milk powders, in particular on oxidative stability of lipid components such as cholesterol. A greater understanding of the changes in whole milk powder during storage arising from changes in pre-heat treatment is required in order to allow selection of optimum and appropriate conditions for manufacture of good quality whole milk powders.

Fat in dried milk products can deteriorate chemically in two ways, (i) the triglycerides can be hydrolysed and the flavour of the product spoiled by excessive liberation of the pungent short chain fatty acids ('hydrolytic rancidity') or (ii) the unsaturated fatty acid constituents of the fat can be oxidised by atmospheric oxygen with the formation of peroxides and ultimately, of aldehydes, acids of low molecular

weight and other compounds of objectionable flavour ('oxidative' rancidity). Since pasteurisation inactivates most lipases associated with 'hydrolytic rancidity', the latter phenomenon is a major problem associated with the long-term storage of whole milk powders (Coulter *et al.*, 1951; Lea, 1953; Shipstead and Tarassuk, 1953; Boon *et al.*, 1976; Tuohy, 1987; Ipsen and Hansen, 1988). As organoleptic examination of milk samples is time-consuming, subjective and expensive, chemical indices of autoxidation are often used, the two most common being peroxide values (PV) and thiobarbituric acid (TBA) determinations (Tuohy, 1987).

Since whole milk powder is subject to oxidation during storage, it is most important that packing precautions are taken to eliminate as much oxygen as possible. There are many different forms of packing materials used for whole milk powder, from tin-plate cans and foil-lined sachets to foil laminates, each with their own specific uses. Tin-plate cans are expensive and there have been developments in flexible film lamination technology (Tuohy, 1984) resulting in a more cost-effective alternative. The main advantages associated with this form of packing are (i) lower cost material, because the metal layer is only 9-12 μm thick, (ii) light weight material for transport and distribution, (iii) flexible construction, allowing a greater weight of powder to be packed per unit load volume. Aluminium foil packs can be made, filled, gas-flushed and sealed on a single machine, occupying a small floor area. Since packs are actually made *in situ,* pack size can be changed, within limits without major disruption. There are, however, some disadvantages associated with using aluminium foil laminates in packing of whole milk powder: (i) laminate packs do not have the mechanical strength and durability of rigid containers, (ii) there can be difficulty getting an airtight pack due to heat-seal failure if the heat area becomes contaminated with powder fines, (iii) as the thickness of the foil used is only 9-12 um, there is the possibility of finding 'pinholes' in the foil layer. Also, during handling of the laminate prior to or during sachet formation, there is a risk of damaging the foil layer and making the laminate permeable to oxygen (Tuohy, 1984). Vacuum-packing reduces the oxygen content of packed whole milk powder by both evacuation of the interstitial air (air between powder particles) and by compression of the powder, which results from vacuumisation (Van Mil and Jans,

1991). Tuohy (1984) found that vacuum-packing in aluminium foil laminates was as effective as gas flushing at retarding lipid oxidation and comparable with canning under nitrogen. An added advantage of vacuum-packing of whole milk powder is the volume reduction arising from the compression which is associated with vacuumisation. Reducing the oxygen content of packed whole milk powder has been reported to significantly reduce oxidation (Tuohy, 1987; Van Mil and Jans, 1991; Chan *et al.*, 1993). De Vilder (1982) reported that when whole milk powder was packed in tins with an atmosphere of nitrogen and carbon dioxide, the flavour score remained favourable for thee years. However, when packed in material which permitted the passage of moisture (paper lined bags) or gas entry (sachets), the organoleptic properties after a period of storage were significantly lower when compared with the initial values.

Autoxidation also depends on temperature: it has been demonstrated that if the temperature is raised by 10° C, the oxidation reaction rate is doubled (Walstra and Jenness, 1984). Oxidation of whole milk powder has been reported to significantly increase at high storage temperatures (Tuohy, 1984; Chan *et al*, 1993). However, an increase in temperature may also lead to an increase in Maillard reactions, resulting in a compensating antioxidative effect of the reaction products (Wyott and Day, 1965; Eriksson, 1982).

Spray-dried, cholesterol-containing foods, subjected to air and heat during processing are susceptible to sterol oxidation during preparation and storage and therefore may benefit from antioxidant addition (Huber *et al.*, 1995). Since cholesterol oxidation proceeds via a free radical mechanism, antioxidants used to inhibit 'general' lipid oxidation may also retard cholesterol oxidation (Smith, 1980; Huber *et al.*, 1995; Lai *et al.*, 1995b). Consumer concerns regarding the safety of synthetic antioxidants, such as BHT has prompted investigations into the effectiveness of naturally occurring compounds, such as the tocopherols (Resurrection and Reynolds, 1990; Huber *et al.*, 1995; Wahle *et al.*, 1993; Li *et al.*, 1996). Nonetheless, studies involving the application of antioxidants to inhibit cholesterol oxidation have been limited. BHA, BHT and propyl gallate, common

synthetic antioxidants, were only slightly effective in slowing hydrogen peroxide-induced cholesterol oxidation in egg yolk during spray-drying (Morgan and Armstrong, 1987). More recent evidence (Rankin and Pike, 1993; Huber *et al.*, 1995) has shown that tocopherol isomers at concentrations of 0.02-0.2 % (w/w total lipid) proved effective in reducing cholesterol autoxidation in spray-dried egg powder. During storage, an inverse relationship between COP formation and α-tocopherol content was observed (Huber *et al.*, 1995).

In Chapter 3, the effects of animal feeding regimen on oxidative stability of whole milk powders were investigated. This chapter deals with an investigation of the effects of pre-heat treatment on oxidative stability of whole milk powders. In addition, the effect of vacuum-packing was compared with sachet-packing for long-term storage of whole milk powder at both ambient (15° C) and 'hot-room' (30° C) temperatures. Therefore, this chapter contains data that overlaps with chapter 3. Finally, a preliminary study was undertaken to investigate the antioxidative effects of α-tocopherol addition to milk both prior to and after spray-drying.

4.2 AIMS

To assess the effects of pre-heat temperature, spray-drying, storage and packing conditions on oxidative stability of whole milk powder.

4.3 OBJECTIVES

- To investigate the effect of manufacture (processing) on the physical, functional and organoleptic properties of whole milk powder.

- To investigate the effects of low pre-heat and high pre-heat temperatures on lipid and cholesterol oxidation in whole milk powder during storage.

- To investigate the effectiveness of reduced oxygen levels in retarding lipid and cholesterol oxidation in whole milk powder. The two packing conditions investigated were sachet-packing and vacuum-packing.

- To investigate the role of storage temperatures on the extent of lipid and cholesterol oxidation in whole milk powder.

- To evaluate the effectiveness of emulsified α-tocopherol in inhibiting lipid and cholesterol oxidation during storage of spray-dried whole milk powder.

4.4 EXPERIMENTAL

4.4.1 Manufacture of whole milk powder

Whole milk powder was manufactured from milk of the grass-fed herd and grass plus concentrate-fed herd in the Moorepark pilot plant as outlined in Chapter 3. Low-heat (75° C for 10 sec) and high-heat (110° C for 2 min) powders were manufactured, and subsequently sachet-packed in aluminium lined paper bags or vacuum-packed in foil laminates and stored at 15° C and 30° C in a walk-in room with artificial lighting and temperature control to ± 2° C.

4.4.2 Manufacture of whole milk powder with α-tocopherol addition

A pilot study was performed in which the effect of α-tocopherol addition on whole milk powder stability was investigated. The α-tocopherol was added to 2 of the manufacturing trials ((3/8/94 (trial 11) and 18/8/94 (trial 12)). The effects of α-tocopherol addition to milk both prior to and following spray-drying were assessed. Standardised milk of the grass-fed herd was subjected to high pre-heat treatment and subdivided into 4 aliquots (50 litres); 1 aliquot was used as control, 2 aliquots for α-tocopherol addition prior to spray-drying and 1 aliquot for α-tocopherol addition after spray-drying. Emulsifed dl-α-tocopherol (oil form, Roche Products Ltd.) was added at two concentrations (500 μg/g lipid and 1000 μg/g lipid) to the milk concentrate on a fat basis prior to spray-drying. The concentrate was homogenised (two-stage) using an APV Manton Gaulin homogeniser (Type KF 3). The homogenised concentrate was spray-dried as outlined in Chapter 3. The powder was collected in a stainless steel bin at the base of the powder recovery cyclone and transferred to foil-sacks. The powder was allowed to cool in the sealed sacks to ambient temperature before packing. Ronoxan D-20 (powder form, Roche Products Ltd.) (a mixture of ascorbyl palmitate, dl-α-tocopherol and citric acid in the proportion 6:2:1, together with an anhydrous glucose and an emulsifier (glyceryl monostearate) was added to 1 aliquot (50 g) of powder at a concentration of 1000 α-tocopherol μg/g on the powder basis for investigation of the effect of α-tocopherol addition after spray-drying.

4.4.3 Whole milk powder analysis

4.4.3.1 Moisture analysis

Samples were analysed for moisture as outlined in Chapter 3, section 3.4.6.1.

4.4.3.2 Determination of fat content in whole milk powder

The fat content of a solution of whole milk powder (10 % w/v) was determined using the Rose-Gottlieb method as outlined in Chapter 3, section 3.4.6.2.

4.4.3.3 Determination of bulk density in whole milk powder

The bulk density of the whole milk powder manufactured in this study was determined according to the IDF (1995) procedure. 100 g whole milk powder was weighed into 250 ml graduated cylinder, which was then placed on the platform of the density tester and 100 jolts applied to the sample. The volume of powder was recorded from the graduated cylinder, and results were expressed in g/ml.

4.4.3.4 Determination of insolubility index in whole milk powder

The insolubility index was determined as outlined in ADMI (1971). A solution of whole milk powder (13 g/100 ml), containing 2-3 drops of antifoam agent was stirred for 90 sec at 24° C and allowed to stand at room temperature for 15 min. The sample was mixed thoroughly for 5 sec and a conical centrifuge tube immediately filled to the 50 ml mark and centrifuged for 5 min at 500 g. The supernatant was then siphoned off to within 5 ml of the surface of the sediment level, taking care not to disturb the sediment layer. 25 ml distilled water at 24° C was added and the tube shaken gently to disperse the sediment, dislodging if necessary with a wire. The tube was again filled to the 50 ml mark with distilled water at 24° C, and inverted several times to mix the contents thoroughly and centrifuged again at 500 g for 5 min. The tube was held in a vertical position and the insolubility index measured to the nearest scale. Results were expressed in ml.

4.4.3.5 Determination of undenatured whey protein nitrogen index

The undenatured whey protein nitrogen index (WPNI) was determined according to the method outlined by the ADMI (1971) as outlined in Chapter 3, section 3.4.6.3.

4.4.3.6 Determination of peroxide values in whole milk powder

The peroxide values (PV) of the powders were assayed according to IDF (1991) as outlined in Chapter 3, section 3.4.6.4. PV values were expressed as mEq O_2/kg fat.

4.4.3.7 Determination of thiobarbituric acid reactive substances in whole milk powder

The thiobarbituric acid reactive substances (TBARS) were determined according to 2-thiobarbituric acid method of Tarladgis *et al.* (1960) as outlined in Chapter 3, section 3.4.6.5. A conversion factor of 6.2 (Crackel *et al.*, 1988) was used to convert absorbance units to mg MDA/kg whole milk powder.

4.4.3.8 Measurement of free sulphydryl groups

The level of free sulphydryl groups was measured using the procedure described by Kalab (1970) and outlined in Chapter 3, section 3.4.6.6. Results were expressed as μmoles/g whole milk powder.

4.4.3.9 Measurement of total sulphydryl groups

Total sulphydryl groups were measured using Ellman's reagent as described by Beveridge *et al.* (1974) and outlined in Chapter 3, section 3.4.6.7. Results were expressed as μmoles/g whole milk powder.

4.4.3.10 Measurement of Vitamin E

Vitamin E concentration in milk powder was determined using the method of Buttriss and Diplock (1984) as outlined in Chapter 3, section 3.4.6.8. Results were expressed in μg/g powder.

4.4.3.11 Analysis of COPs

COPs were analysed using the method outlined in Chapter 2, sections 2.4.4 and 2.4.5 and results expressed as μg/g lipid.

4.4.3.12 Organoleptic Assessment of whole milk powder

Organoleptic assessment was carried out by The Borden's Company Ltd. (see appendix III for detailed procedure) on a sub-sample of powders to investigate the effect of pre-heat temperature, packing and storage conditions on organoleptic properties of powders using an in-house method described as follows: 13 g whole milk powder was reconstituted by addition of 90 ml demineralised water (25° C) and mixed in a blender for 90 sec. The reconstituted sample was allowed to stand for one h at room temperature before tasting. Panellists tasted the samples to which they assigned a score on their flavour score sheet (appendix III). The flavour score was reported by a numbering system as follows:

90: Perfect, slightly cooked flavour.
85: Good: no off-flavour, not quite as fresh tasting as 90.
80: Fair; no strong off-flavour, but less fresh tasting then 85.
75: Objectionable; some off-flavour.

'Off flavours' were identified as:

(i) Acid
(ii) Bitter
(iii) Chalky
(iv) Burnt
(v) Feed
(vi) Flat
(vii) Metallic
(viii) Oxidised or tallowy
(ix) Rancid
(x) Salty
(xi) Stale
(xii) Weedy

4.4.4 Statistical Methods

Results were statistically analysed as outlined in Chapter 2, section 6, with correlation coefficients determined by Pearson's Correlations using the SYS-STAT package.

4.5 RESULTS

4.5.1 Physical, chemical and functional characteristics of manufactured whole milk powder

Whole milk powder was manufactured on 14 different occasions thoughout the lactation period. All milks were heat treated prior to powder manufacture, equal volumes (50 litres) being subjected to low-heat (75° C for 10 sec) treatment and to high-heat (110° C for 2 min) treatment. The powders were analysed for physical, functional and organoleptic properties prior to and after storage for 12 months at 'ambient' (15° C) temperature and 'hot room' (30° C) temperature. Table 4.1 describes the chemical composition and physical, functional and organoleptic properties of fresh and stored whole milk powders. Neither pre-heating nor storage had an effect on moisture content of whole milk powders. There was no significant difference between the moisture contents of freshly manufactured low-heat powders (2.01 $\pm$ 0.39 %) and high-heat powders (2.93 $\pm$ 0.80 %). Similarly, there was no significant difference stored low-heat (1.99 $\pm$ 0.45 %) and high-heat whole milk powders (2.78 $\pm$ 0.45 %). Bulk density of low- and high-heat whole milk powders ranged from 0.51 to 0.56 g/ml. No significant difference was observed in this parameter in fresh or stored powders.

A difference in the insolubility index, though not significant, was observed between low-heat and high-heat whole milk powders. Freshly manufactured low-heat powders had a mean insolubility index of 0.1 $\pm$ 0.23 ml compared to 0.4 $\pm$ 0.36 ml in fresh high-heat powders. Ambient temperature storage increased the insolubility index in both low-heat (approximately 3-fold) and high-heat powders (approximately 1.5-fold). Storage at 30° C increased the insolubility index in low- and high-heat powders approximately 5-fold and 1.5-fold, respectively.

The fat content of the powders (Table 4.1) was not affected by heat treatment or by storage. Freshly manufactured whole milk powders showed PV levels ranging from 0.40 - 0.43 mEq O_2/kg and TBARS levels ranging from 0.08 - 0.11 MDA/kg.

Table 4.1. Properties of fresh and stored high-heat and low-heat whole milk powders[n] of the grass-fed herd. All powders were sachet-packed and stored at 15 and 30° C for 12 months. Data refers to mean ± standard deviation.

Whole Milk Powder Characteristics	**Fresh low-heat whole milk powder**	**Fresh high-heat whole milk powder**	**12 month old low-heat whole milk powder (15 °C)**	**12 month old low-heat whole milk powder (30°C)**	**12 month old high-heat whole milk powder (15 °C)**	**12 month old high-heat whole milk powder (30 °C)**
Moisture (%)	2.01 ± 0.39[a]	2.93 ± 0.80[a]	1.99 ± 0.45[a]	2.02 ± 0.45[a]	2.78 ± 0.45[a]	2.69 ± 0.45[a]
WPN index (mg/g)	6.90 ± 0.83[a]	1.30 ± 0.20[a]	6.60 ± 0.65[b]	6.49 ± 0.45[b]	1.10 ± 0.45[b]	1.29 ± 0.36[b]
Fat (%)	26.28 ± 0.26[a]	26.11± 0.84[a]	25.12 ± 0.45[b]	25.99 ± 0.41[b]	25.99 ± 0.65[b]	25.60 ± 1.05[b]
Bulk density (g/ml)	0.55 ± 0.34[a]	0.55 ± 0.52[a]	0.54 ± 0.35[b]	0.54 ± 0.45[b]	0.56 ± 0.46[b]	0.51 ± 0.09[b]
Insolubility index (ml)	0.10 ± 0.23[a]	0.40 ± 0.36[a]	0.30 ± 0.54[b]	0.53 ± 0.45[b]	0.60 ± 0.32[b]	0.58 ± 0.43[b]
Peroxide Value (mEq/kg fat)	0.43 ± 0.14[a]	0.40 ± 0.09[a]	1.99 ± 0.60[a]	2.72 ± 0.95[a]	1.17 ± 0.73[a]	1.74 ± 0.62[a]
TBA (mg MDA/kg)	0.11 ± 0.04[a]	0.08 ± 0.02[a]	0.53 ± 0.15[a]	1.59 ± 0.45[a]	0.29 ± 0.09[a]	0.44 ± 0.09[a]
Flavour	85 ± 2.5[b]	85 ± 3.7[b]	82 ± 3.2[b]	75 ± 3.2[b]	90 ± 4.1[b]	80 ± 3.98[b]

[a] n = 14 [b] n = 4

n= number of samples analysed

Storage for 12 months at 15° C resulted in a significant increase ($p < 0.01$) in PV values in low-heat powders (1.99 O_2/kg fat) and high-heat powders (1.17 mEq O_2/kg fat), and a similarly significant ($p < 0.01$) increase in PV levels was obtained at 'hot room' storage temperatures compared to storage at 15° C. TBARS were also significantly higher ($p < 0.01$) in low and high-heat whole milk powders at both 15 and 30° C. Organoleptic evaluation of whole milk powder showed that freshly manufactured powders had a flavour score of 85, which was classified by trained panellists as 'good'. However, the flavour score deteriorated to 82 ('slightly oxidised') in stored low-heat powders and increased in the high-heat powders to 90 ('perfect flavour').

The effects of animal feeding regimen and pre-heating temperatures on WPNI values of whole milk powders are shown in Fig. 4.1. Animal feed regimen resulted in significantly higher levels of undenatured whey protein during period 1 ($p < 0.05$) but had no significant effect on powders produced in periods 2 and 3. The low-heat powders of the herds R and S had mean WPNI values of 6.9 ± 0.8 and 7.8 ± 1.1 mg undenatured whey protein/g powder, respectively, compared to values of 1.3 ± 0.2 and 1.1 ± 0.2 mg undenatured whey protein/g powder, respectively, for corresponding high-heat powders. All low-heat and high-heat powders manufactured thoughout the study fell within the limits of each class according to the WPNI. As expected, the high pre-heating temperature (110° C) led to significantly more protein denaturation (Fig. 4.1) than the low pre-heating temperature (75° C).

The levels of 'free' sulphydryl groups found in milk powder used in this study are compared in Table 4.2. High pre-heat treatment of milk from both herds resulted in increased levels of 'free' sulphydryl groups ($p < 0.05$) compared with low pre-heat treatment. The 'free' sulphydryl content of powders manufactured from milk of the grass plus concentrate-fed herd (S) were significantly ($p < 0.05$) higher than the grass-fed herd (R) at both low and high-pre-heat temperatures. High-heat powder of the grass-fed herd yielded, on average, 0.28 ± 0.04 μmoles 'free' sulphydryl

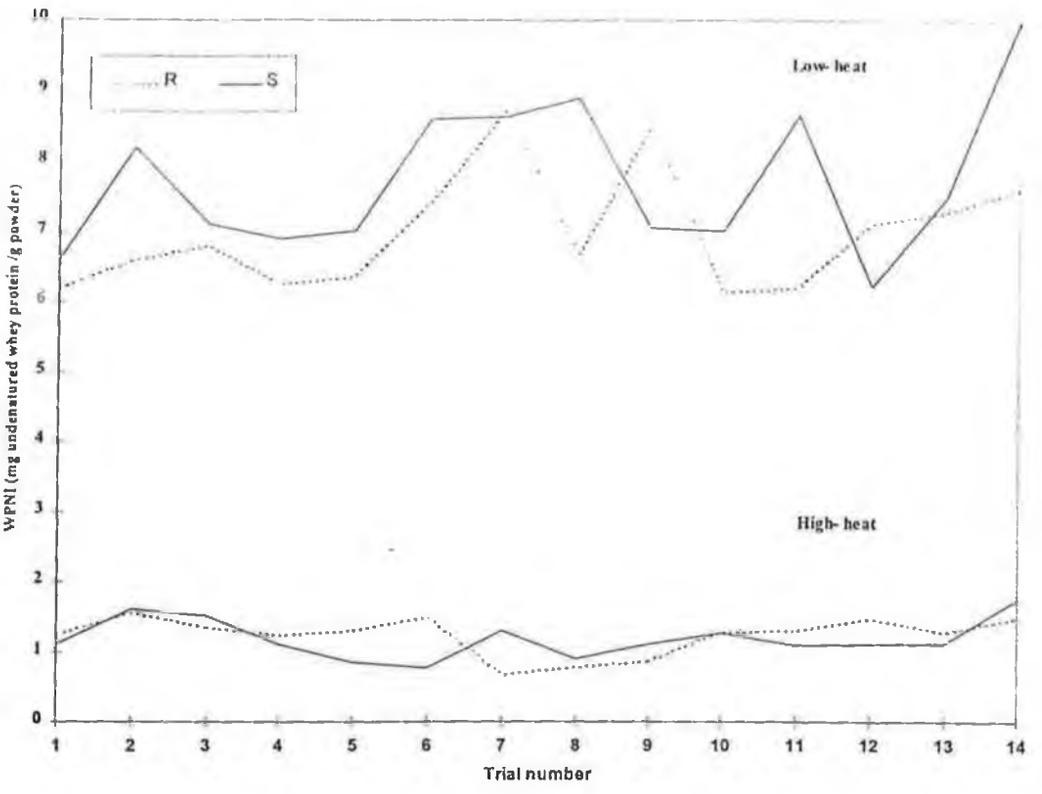


Fig. 4.1 WPNI of low and high-heat whole milk powders of grass-fed herd (R) and grass plus concentrate-fed herd (S).

groups/g powder, while corresponding low-heat powders yielded only 0.07 ± 0.02 μmoles/g powder. The inverse was found for 'total' sulphydryl levels, which decreased as the pre-heat temperature increased. The level of 'total' sulphydryl groups for low-heat powder of the grass-fed herd was 15.9 ± 0.03 μmoles/g compared to 0.14 ± 0.03 μmoles/g for high-heat powder of the same herd. Total sulphydryl levels in high-heat powders were, however, observed to be lower than the free sulphydryl content, suggesting that heat-treatment adversely affected the ability of mercaptoethanol to reduce disulphide groups in high-heat powders.

Table 4.2. Free and total sulphydryl group concentration in 12 month stored whole milk powder[n].

Herd	**Heat class**	**Free sulphydryl μmoles/g powder**	**Total sulphydryl μmoles/g powder**
R	low	0.07 ± 0.02	15.91 ± 0.03
S	low	0.11 ± 0.04 [c]	16.88 ± 0.03
R	high	0.28 ± 0.04 [a]	0.14 ± 0.03
S	high	0.46 ± 0.04 [b, d]	0.16 ± 0.04

n (number of samples analysed)=7

[a] $p < 0.05$ relative to grass-fed grass-fed, low-heat class

[b] $p < 0.05$ relative to grass plus concentrate-fed herd S, low heat class

[c] $p < 0.05$ relative to grass-fed grass-fed, low-heat class

[d] $p < 0.05$ relative to grass-fed grass-fed, high heat class

4.5.2 Effect of pre-heat treatment on oxidative stability during storage of whole milk powder.

The data from this study indicates that high pre-heating conditions led to improved oxidative stability in whole milk powders during subsequent storage compared to low pre-heat treatment. The PV values obtained for low-heat powders were significantly higher ($p < 0.05$) than those of high-heat powders after 6 months storage at the temperatures examined (15° C and 30° C; Fig. 5.2 a-d, broken lines). While PVs increased 7-fold in low-heat powders, which were sachet-packed and stored at 15° C for 6 months, an increase of only 3.5-fold was observed in high-heat powders under similar packaging and storage conditions (Fig. 5.2 a). Similarly, TBARS levels were significantly reduced ($p < 0.01$) in high-heat powders compared to low-heat powders during the 12-month storage period (Fig. 5.2 a-d solid lines). When PV levels were correlated with storage time, PV levels reached a maximum at 6 months storage with correlation coefficients of 0.92 ± 0.07. TBARS levels were found to reach a maximum at 12 months, with correlation coefficients of 0.94 ± 0.05, when corrleated with time, indicating a progression from a primary to secondary oxidation state.

4.5.3 Effect of packing on oxidative stability of whole milk powder.

Vacuum-packing of whole milk powder resulted in significantly lower PV and TBARS values compared to sachet-packing ($p < 0.001$) during subsequent storage at 15° C and 30° C (Fig. 4.2 c-d). Vacuum-packing reduces the oxygen content of packed powders by both evacuation of the interstitial air and compression of the powder (Tuohy, 1984). In the present study, TBARS values of sachet-packed powders of the grass-fed herd increased 5-fold during storage for 12 months at 15°C (Fig. 4.2 a) and 15-fold during storage at 30° C (Fig. 4.2 b), while only 3 and 4-fold increases were observed when powders were vacuum-packed and stored at 15° C and 30° C (Fig. 4.2 c-d), respectively.

4.5.4 Effect of storage temperature on oxidative stability of whole milk powder.

Storage temperature played a significant role in lipid oxidation of milk powder in the present study, with higher storage temperatures leading to significantly increased PV

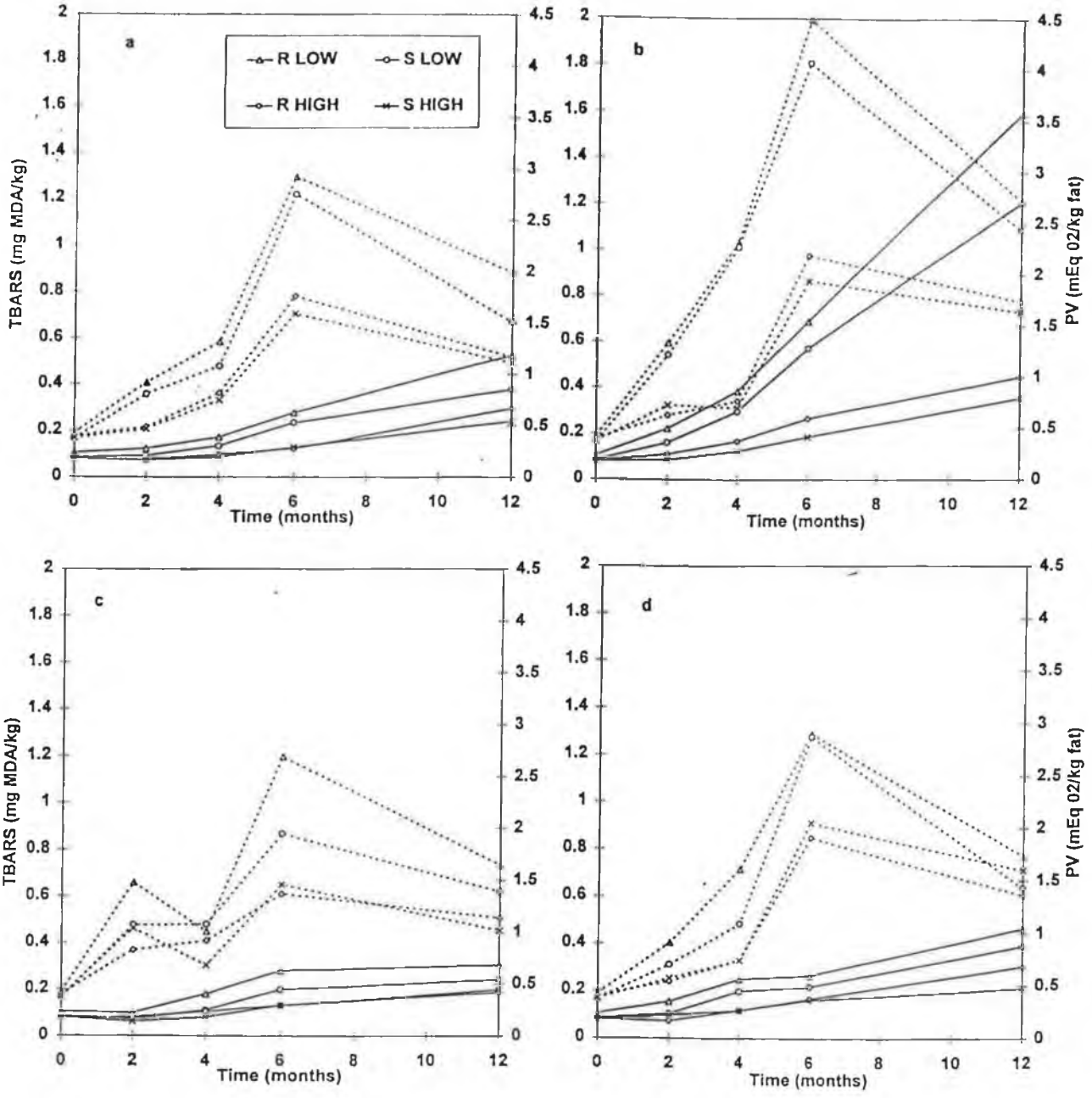


Fig. 4.2 PV (---) and TBARS (_) were monitored in high-heat (110° C for 2 min) and low-heat (75° C for 10 sec) whole milk powders, manufactured from milk of grass-fed herd (R) and grass plus concentrate-fed herd (S).

a. Sachet-packed and stored at 15° C; b. Sachet-packed and stored at 30° C;
c. Vacuum-packed and stored at 15° C; d. Vacuum-packed and stored at 30° C.

R LOW low-heat powder of the grass only-fed herd

R HIGH high-heat powder of the grass only-fed herd

S LOW low-heat powder of the concentrate plus grass-fed herd

S HIGH high-heat powder of the concentrate plus grass-fed herd

levels ($p < 0.001$) and TBARS values ($p < 0.001$). Fig. 4.2 (a) illustrates the levels of PV (broken lines) and TBARS (solid lines) obtained from sachet-packed low-heat and high-heat whole milk powders of the herds R and S and stored at 15° C for up to 12 months, while Fig. 4.2 (b) shows similar powders, but stored at 30° C. PV levels increased at an accelerated rate compared to TBARS, reaching a maximum at 6 months storage at 15° C and 30° C for all powders investigated, and declined thereafter (Fig. 4.2 a-d). An inverse relationship was observed between PV and TBARS after 6 months storage at both 15° C and 30° C, indicating progression of oxidation from a primary to a secondary state. While a 7-fold increase in PV values was observed due to storage of low-heat powders at 15° C for 6 months, storage at 30° C resulted in a 10-fold increase in PV values (Fig. 4.2 b). A greater difference between storage temperatures was noted in low-heat ($p < 0.001$) than high-heat powders ($p < 0.05$), i.e. a significant interaction ($p < 0.01$) between pre-heat and storage temperature existed. Table 4. 3 summarises the interactions which occurred between treatments during this study. Significant interactions were found between packing conditions and animal feed quality ($p < 0.05$), storage temperature and animal feed quality ($p < 0.05$), packing conditions and pre-heat temperature ($p < 0.01$), storage temperature and pre-heat temperature ($p < 0.05$) and storage temperature and packing conditions ($p < 0.001$). This data indicates that the effect of any one of these treatments on the oxidative stability of whole milk powder was increased when combined with another treatment, e.g. the effect of sachet-packing on oxidative stability of whole milk powder was greater at the higher storage temperature of 30° C than at the ambient storage temperature of 15° C.

Table 4.3 Interactions between animal feed quality, pre-heat temperature, packing and storage temperature. Values refer to probabilities.

Effect	Animal Feeding Regimen	Pre-heat	Packing	Storage Temperature
Animal Feeding Regimen	-	N.S.	0.05	0.05
Pre-heat	N.S.	-	0.01	0.05
Packing	0.05	0.01	-	0.001
Storage Temperature	0.05	0.05	0.001	-

4.5.5 Effect of processing, packing and storage on organoleptic properties of whole milk powder.

Table 4.4 shows the data obtained by sensory evaluation of the low and high-heat whole milk powders of the grass-fed and grass plus concentrate-fed herd after 12 months storage at 15 and 30° C. Low-heat whole milk powder stored at 30° C obtained the lowest flavour score (75), indicative of oxidised flavour. While storage of low-heat and high-heat powders at 15° C resulted in slightly oxidised flavour score of 80-82, the highest flavour score was obtained for high-heat vacuum-packed powders stored at 15° C which resulted in a flavour score of 90 and was deemed 'acceptable' by taste panellists. Statistical analyses showed no significant differences between whole milk powders of both herds. However, after 12 months storage at 15° C, vacuum-packed powders of the grass-fed herd resulted in a 'strongly oxidised' flavour, while the corresponding powders from the grass plus concentrate-fed herd gave rise to an 'oxidised' flavour.

Table 4. 4 Sensory evaluation of stored low and high whole milk powders.

Heat Treatment	Animal feeding Regimen	Packing type	Storage temp. (° C)	Flavour score	Comments
Low-heat	Grass only	Sachet	15	82	Slightly oxidised
	Grass plus concentrate	Sachet	15	84	Slightly oxidised
Low-heat	Grass only	Sachet	30	75	Strongly oxidised
	Grass plus concentrate	Sachet	30	76	Strongly oxidised
Low-heat	Grass only	Vacuum	15	80	Oxidised
	Grass plus concentrate	Vacuum	15	82	Slightly oxidised
Low-heat	Grass only	Vacuum	30	75	Strongly oxidised
	Grass plus concentrate	Vacuum	30	80	Oxidised
High-heat	Grass only	Sachet	15	82	Slightly oxidised
	Grass plus concentrate	Sachet	15	83	Slightly oxidised
High-heat	Grass only	Sachet	30	80	Oxidised
	Grass plus concentrate	Sachet	30	80	Oxidised
High-heat	Grass only	Vacuum	15	90	Good
	Grass plus concentrate	Vacuum	15	90	Good
High-heat	Grass only	Vacuum	30	82	Slightly oxidised
	Grass plus concentrate	Vacuum	30	84	Slightly oxidised

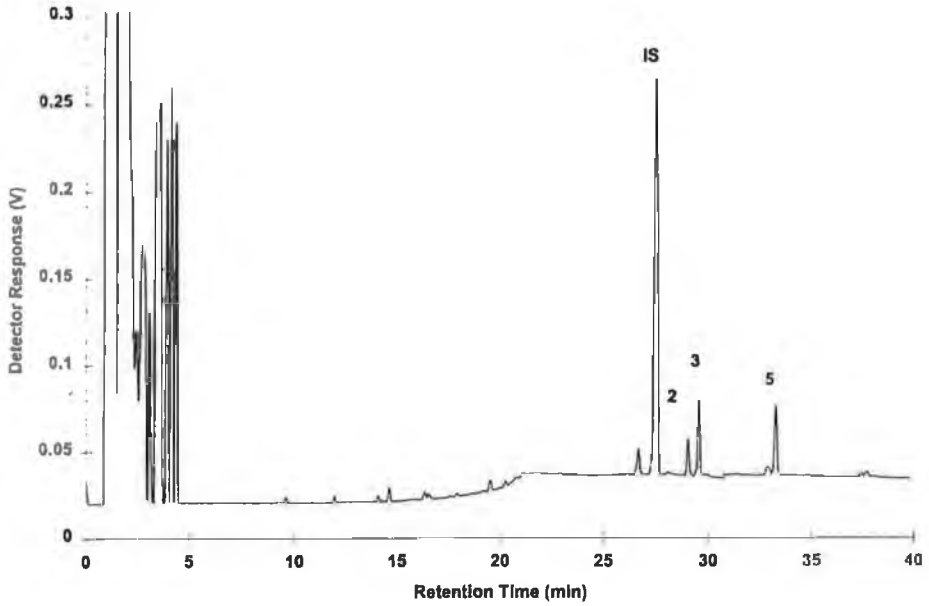
4.5.6 Effect of pre-heat, packing and storage temperature on cholesterol oxidation in whole milk powder of the grass-fed and grass plus concentrate-fed herds

In the present study, 'total COPs' refers to the quantification of 5 of the most commonly found COPs in processed foods, which are the cholesterol-α- and β-epoxides, 7-ketocholesterol, 25-hydroxycholesterol, and cholestanetriol. Fig. 4.3 shows a chomatogram of stored sachet-packed whole milk powder from (a) grass only and (b) grass plus concentrate-fed herds. The most notable difference in these chomatograms is the presence of cholestanetriol in the powder of the herd fed grass only, but not in the powder of the herd fed grass plus concentrate. Fig. 4.4 shows the levels of total COPs in low- and high-heat whole milk powders manufactured from both herds. The histogram depicts the total COPs data obtained from fresh powders and vacuum and sachet-packed powders stored at 15° C and 30° C for 12 months.

In freshly prepared milk powders, high pre-heat treatment resulted in significantly higher levels ($p < 0.001$) of COPs than low pre-heat treatment. The total COPs content of fresh high-heat powders from the grass-fed herd was 1.6 ± 0.55 μg/g lipid, compared with 0.66 ± 0.24 μg/g lipid for low-heat powders (Fig. 4.4). COPs levels of the corresponding high-heat and low-heat powders of the grass plus concentrate-fed herd were 1.07 ± 0.60 and 0.43 ± 0.24 μg/g lipid, respectively. Thus, the higher plane of nutrition offered a protective effect against cholesterol oxidation during whole milk powder processing.

Low-heat powders from the grass-fed herd which were sachet-packed and stored at 15° C had 1.9-fold higher COPs (5.5 ± 1.3 μg/g lipid) than high-heat powders produced from the grass-fed herd and stored under the same conditions (2.9 ± 1.2 μg/g lipid). In addition, low-heat powders of the grass-fed herd had 1.4-fold higher COPs levels compared to similarly prepared powders from the grass plus concentrate-fed herd (3.9 ± 1.0 μg/g lipid). Under these conditions (i.e. sachet-packing and storage at 15° C), the lowest levels of COPs were obtained in high-heat powders manufactured from the grass plus concentrate-fed herd (1.7 ± 0.7 μg/g lipid) (Fig. 4.4).

(a)



(b)

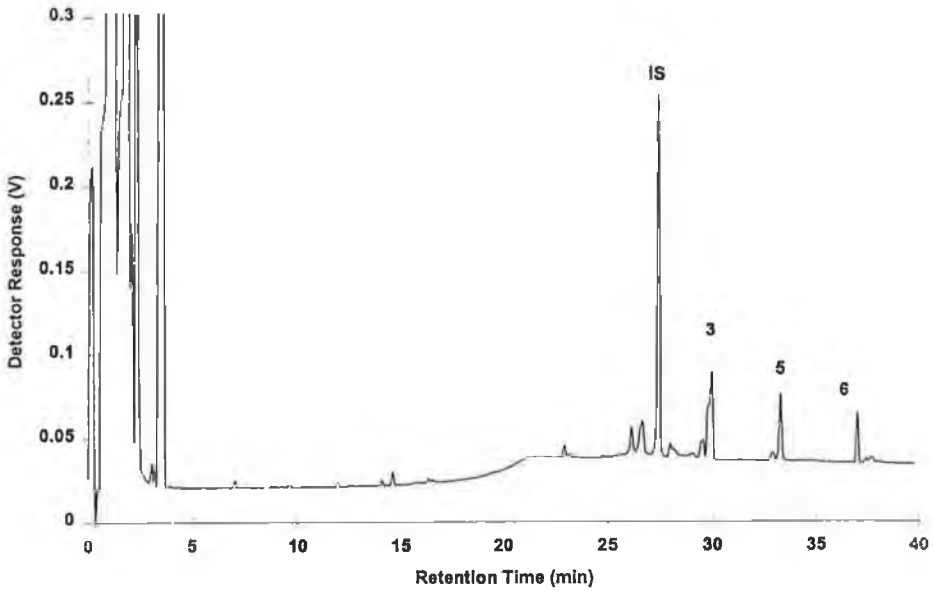


Fig. 4.3 Chomatogram of sachet-packed low-heat whole milk powder of the (a) grass-fed herd (R) and (b) grass plus concentrate-fed herd (S) stored for 12 months at 30° C. IS: internal standard; 2: cholesterol-β-epoxide; 3: cholesterol-α-epoxide; 5: 7-ketocholesterol and 6 :cholestanetriol.

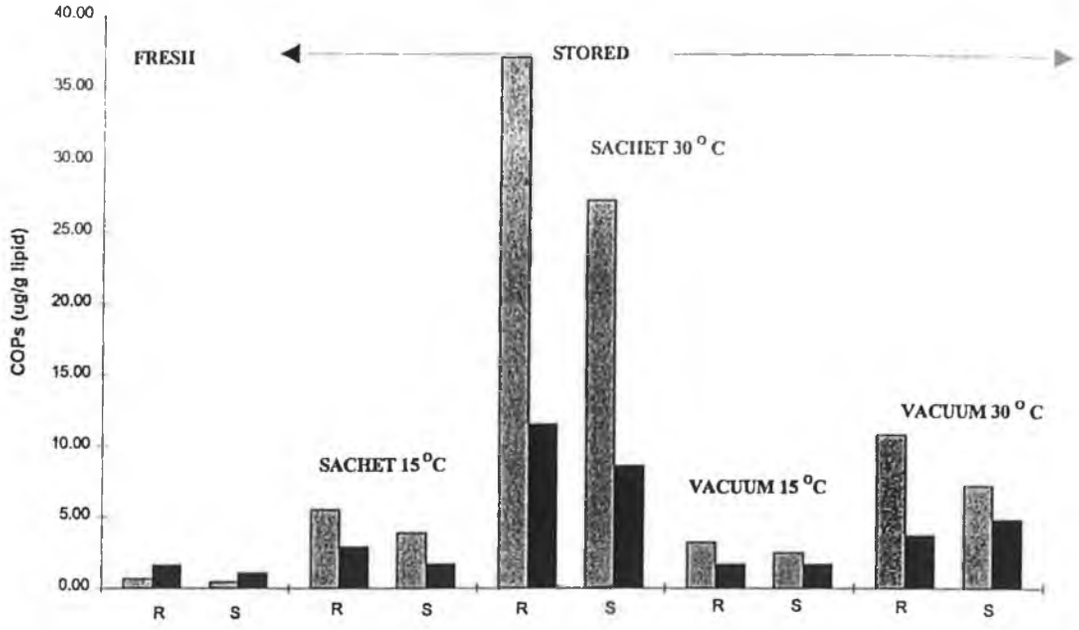


Fig. 4.4 Total COPs in fresh and stored whole milk powder of the (a) grass-fed herd (R) and (b) grass plus concentrate-fed herd (S), sachet and vacuum packed and stored at 15 and 30° C for 12 months.

Low-heat powders from grass-fed that were sachet-packed and stored at 30° C for 12 months had 3.2-fold higher COPs (37.2 ± 7.5 μg/g lipid) than sachet-packed high-heat powders produced from the same herd (11.5 ± 2.4 μg/g lipid) and stored at 30° C for 12 months. In addition, low-heat powders from the grass-fed herd had 1.4-fold higher COPs compared with similarly prepared powders from herd S (27.2 ± 6.7 μg/g lipid) (Fig. 4.4). Under these conditions (i.e. sachet-packing and storage at 30° C), the lowest levels of COPs was obtained in high-heat powders manufactured from the grass plus concentrate-fed herd (8.6 ± 3.4 μg/g lipid).

Low-heat powders from grass-fed that were vacuum-packed and stored at 15° C for 12 months had 1.7-fold higher COPs (3.2 ± 0.8 μg/g lipid) than similarly stored high-heat powders produced from the same herd (1.7 ± 0.6 μg/g lipid). In addition, these low-heat powders from grass-fed had 1.7-fold higher COPs compared with similarly prepared powders from herd S (2.5 ± 0.7 μg/g lipid). Under these storage conditions (i.e. vacuum-packing and storage at 15° C), the lowest level of COPs was obtained in high-heat powders manufactured from the grass plus concentrate-fed herd S (1.69 ± 0.61 μg/g lipid).

Low-heat powders from grass-fed, vacuum-packed and stored at 30° C for 12 months had 2.2-fold higher COPs levels (10.8 ± 3.8 μg/g lipid) than similarly packed and stored high-heat powders produced from the same herd (4.8 ± 1.8 μg/g lipid). In addition, these low-heat powders produced from grass-fed had 1.5-fold higher COPs compared with similarly prepared powders from herd S (7.2 ± 1.5 μg/g lipid). Under these storage conditions (i.e. vacuum-packing and storage at 30° C), the lowest level of COPs was observed in high-heat powders manufactured from the grass plus concentrate-fed herd S (3.7 ± 1.9 μg/g lipid).

In the present study, cholestanetriol and 25-hydroxycholesterol were not detected in powders stored at 15° C (Table 4.5 and Table 4.7), but were detected in powders stored at 30° C (Table 4.6 and Table 4.8). 7-Ketocholesterol, cholesterol-α-epoxide and cholesterol-β-epoxide were all significantly ($p < 0.01$) higher in powders stored at 30° C compared to storage at 15° C. Interestingly, all five COPs investigated were

detected in measurable amounts only in sachet-packed low-heat powders manufactured from milk of the grass-fed grass-fed and stored for 12 months at 30° C. Under these conditions, the following oxides appeared in decreasing order: 7-ketocholesterol > cholesterol-β-epoxide > cholestereol-α-epoxide > 25-hydroxycholesterol > cholestanetriol. 7-Ketocholesterol and cholesterol-β-epoxide were significantly ($p < 0.05$) higher in sachet-packed whole milk powder of grass-fed compared to herd S stored at 15° C (Table 4.5). 7-Ketocholesterol, 25-hydroxycholesterol and cholesterol-β-epoxide were significantly ($p < 0.05$) higher in sachet-packed whole milk powder of grass-fed and herd S stored at 30° C compared to sachet-packed powders stored at 15° C (Table 4.6). Cholesterol-α-epoxide was detected in vacuum-packed whole milk powder from the grass herd but not in that from the milk of herd S stored at 15° C (Table 4.7). 7-Ketocholesterol, 25-hydroxycholesterol and cholesterol-β-epoxide were significantly ($p < 0.05$) higher in vacuum-packed whole milk powder of grass-fed compared to whole milk powders of herd S stored at 30° C (Table 4.8).

The concentration of C_7 oxidation products (primary oxidation products) represented greater than 50 % and 76 % of the COPs present in powders stored at 15° C and 30° C, respectively. This suggests that oxidation products at the allylic position of cholesterol , i.e. C_7 , are formed more abundantly than those at the double bond itself, i.e. positions 5 and 6. Secondary oxidation products (the cholesterol α- and β-epoxides, 25-hydroxycholesterol and cholestanetriol) were present in greater amounts at the high storage temperature of 30° C. Thus, the higher storage temperature appeared to accelerate cholesterol oxidation. A linear relationship was observed between lipid oxidation, quantified by the TBA method and cholesterol oxidation with correlation coefficients of 0.73 ($p < 0.001$) and 0.55 ($p < 0.001$) for sachet-packed and vacuum-packed powders, respectively (Fig. 4.5 a and b). This indicates that the oxidative rate of cholesterol during storage of whole milk powder coincides with TBARS and is more highly correlated for sachet-packed powders.

Table 4.5 COPs in sachet-packed low-heat and high-heat whole milk powders* of grass-fed herd (R) and grass plus concentrate-fed herd (S) stored at 15° C for 12 months.

Heat Class	7-Keto	25-OH	α-epoxy	β-epoxy	Triol	Total COPs (μg/g lipid)	Total COPs (% cholesterol)
Low R	4.18 ± 0.94 [a]	-	0.65 ± 0.11	0.67 ± 0.9 [b]	-	5.50 ± 1.27 [a, b]	0.27 ± 0.06 [a, b]
Low S	3.42 ± 0.8 [d]	-	0.47 ± 0.08 [d]	-	-	3.89 ± 1.01 [d]	0.19 ± 0.10 [d]
High R	2.11± 0.34 [c]	-	0.34 ± 0.09	0.46 ± 0.11 [c]	-	2.91 ± 1.18 [c]	0.15 ± 0.06 [c]
High S	1.46 ± 0.21	-	0.25 ± 0.13	-	-	1.71 ± 0.69	0.09 ± 0.04

[a] $P < 0.05$ relative to high-heat powder from grass-fed grass-fed

[b] $P < 0.05$ relative to low-heat powder from grass plus concentrate-fed herd S

[c] $P < 0.05$ relative to high-heat powder from grass plus concentrate-fed herd S

[d] $P < 0.01$ relative to high-heat powder from grass plus concentrate-fed herd S

* Mean ± SD of 14 samples

Table 4.6 COPs in sachet-packed low-heat and high-heat whole milk powders* of grass-fed herd (R) and grass plus concentrate-fed herd (S) stored at 30° C for 12 months.

Heat Class	7-Keto	25-OH	α-epoxide	β-epoxide	Triol	Total COPs (μg/g lipid)	Total COPs (% cholesterol)
Low R	19.32 ± 4.10 [a, b]	1.32 ± 0.64 [a]	3.8 ± 0.50 [a, e]	11.7 ± 2.40 [a, b]	0.98 ± 0.31 [a, e]	37.17 ± 7 54 [a, e]	1.86 ± 0.57 [a, e]
Low S	15.43 ± 1.84 [d]	1.92 ± 0.21 [d]	0.64 ± 0.41 [d]	9.23 ± 0.29 [d]		27.22 ± 6.70 [d]	1.36 ± 0.12 [d]
High R	7.88 ± 1.43 [c]	-	1.58 ± 0.33	2.06 ± 0.59	-	11.52 ± 2.35 [c]	0.58 ± 0.12 [c]
High S	5.13 ± 1.13	-	1.60 ± 0.41	1.87 ± 0.41	-	8.60 ± 3.40	0.43 ± 0.17

[a] $P < 0.05$ relative to high-heat powder from grass-fed grass-fed

[b] $P < 0.05$ relative to low-heat powder from grass plus concentrate-fed herd S

[c] $P < 0.05$ relative to high-heat powder from grass plus concentrate-fed herd S

[d] $P < 0.01$ relative to high-heat powder from grass plus concentrate-fed herd S

[e] $P < 0.01$ relative to low-heat powder from grass plus concentrate-fed herd S

* Mean ± SD of 14 samples

Table 4.7 COPs in vacuum-packed low-heat and high-heat whole milk powders* of grass-fed herd (R) and grass plus concentrate-fed herd (S) stored at 15° C for 12 months.

Heat Class	7-Keto	25-OH	α-epoxide	β-epoxide	Triol	Total COPs (μg/g lipid)	Total COPs (% cholesterol)
Low R	2.92 ± 0.49	-	0.27 ± 0.09 [a, b]	-	-	3.19 ± 0.85 [a, b]	0.16 ± 0.04 [a, b]
Low S	2.49 ± 0.71	-	-	-	-	2.49 ± 0.71	0.12 ± 0.09
High R	1.90 ± 0.38	-	-	-	-	1.90 ± 0.38	0.10 ± 0.03
High S	1.69 ± 0.61	-	-	-	-	1.69 ± 0.61	0.09 ± 0.03

[a] $P < 0.05$ relative to high-heat powder from grass-fed grass-fed

[b] $P < 0.05$ relative to low-heat powder from grass-fed grass-fed

* Mean ± SD of 14 samples

Table 4.8 COPs in vacuum-packed low-heat and high-heat whole milk powders* of grass-fed herd (R) and grass plus concentratefed herd (S) stored at 30° C for 12 months.

Heat Class	7-Keto	25-OH	α-epoxide	β-epoxide	Triol	Total COPs (μg/g lipid)	Total COPs (% cholesterol)
Low R	6.24 ± 0.86 [a, b]	1.77 ± 0.67 [a, e]	-	2.33 ± 0.99 [a, b]	0.44 ± 0.08 [a, e]	10.79 ± 3.78 [a, e]	0.54 ± 0.19 [a, e]
Low S	4.21 ± 0.99	0.69 ± 0.54 [d]	-	1.32 ± 0.84	-	7.22 ± 1.55 [d]	0.36 ± 0.17 [d]
High R	3.69 ± 0.38[c]	-	-	1.14 ± 0.13	-	4.83 ± 1.79 [c]	0.24 ± 0.09 [c]
High S	2.81 ± 0.29	-	-	0.92 ± 0.19	-	3.73 ± 1.88	0.19 ± 0.09

[a] $P < 0.05$ relative to high-heat powder from grass-fed grass-fed

[b] $P < 0.05$ relative to low-heat powder from grass plus concentrate-fed herd S

[c] $P < 0.05$ relative to high-heat powder from grass plus concentrate-fed herd S

[d] $P < 0.01$ relative to high-heat powder from grass plus concentrate-fed herd S

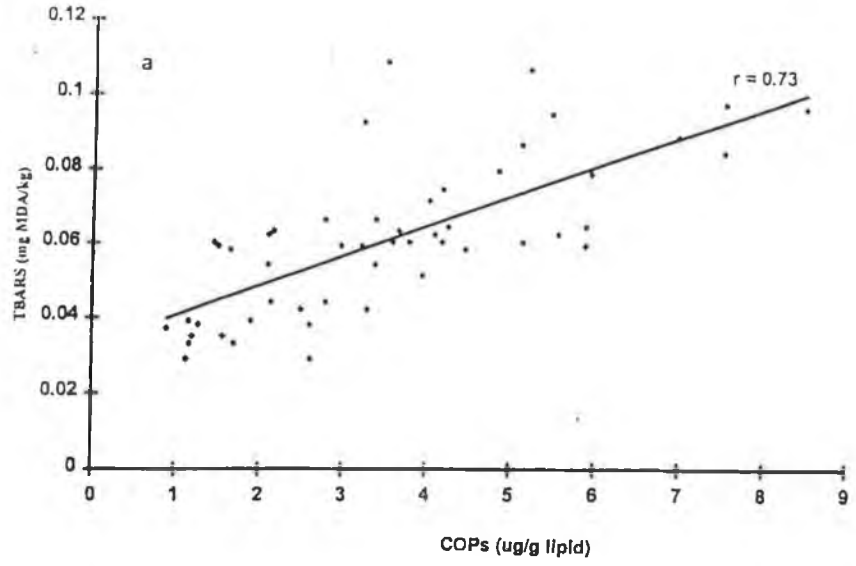
[e] $P < 0.01$ relative to low-heat powder from grass plus concentrate-fed herd S

* Mean ± SD of 14 samples

4.5.7 Effect of α-tocopherol addition on oxidative stability of whole milk powder

Fig. 4.6 shows the α-tocopherol levels in fresh and 12-month-old stored high-heat whole milk powder. Endogenous vitamin E levels of control fresh milk powder (11.08 μg/g) to which no extra vitamin E was added was approximately 2-fold greater than in 12 month old powders (5.42 μg/g). Fresh milk powders produced after addition of 500 μg/g α-tocopherol on a milk fat basis prior to spray-drying contained 150 μg/g vitamin E, which decreased by 16 % upon storage for 12 months. Fresh milk powders produced after addition of 1000 μg/g α-tocopherol to the milk prior to spray-drying contained 324 μg/g vitamin E which did not appreciably decline after storage for 12 months (Fig. 4.6). This data indicates that when α-tocopherol (500 μg/g and 1000 μg/g) was added to milk prior to spray-drying approximately 70 % was lost as a result of whole milk powder manufacture (Fig. 4.6). However, greater than 80 % of the vitamin E was retained in the milk powder over a 12 month storage period in sachet-packed powders stored at 15° C. When 1000 μg/g dried α-tocopherol was added to freshly manufactured whole milk powder on powder basis, the levels of vitamin E detected were not significantly different from control powders to which no antioxidant was added (Fig. 4.6). This may have been due to localised mixing only or inadequate sampling.

(a)



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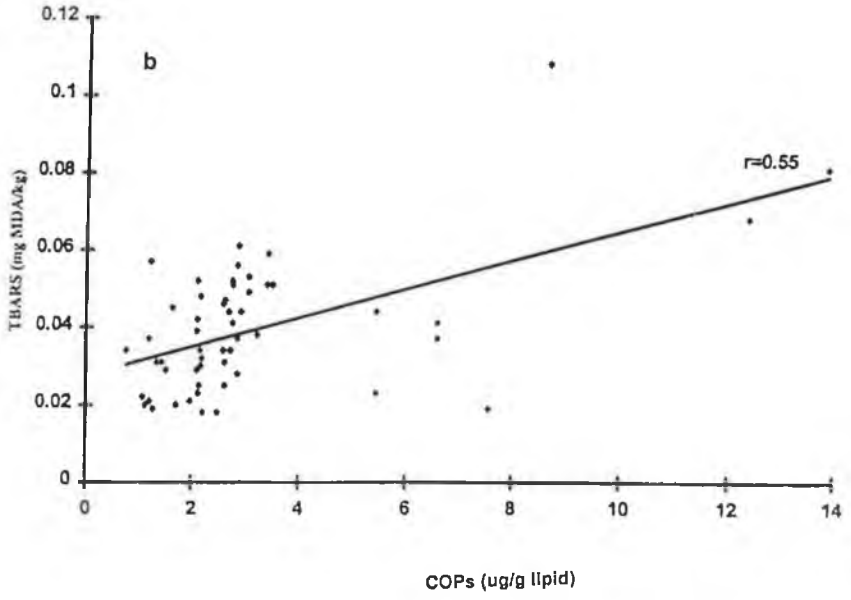

Fig. 4.5 Correlation between COPs and TBARS in stored (12 months) whole milk powders (a) sachet and (b) vacuum-packed at 15 and 30 °C.

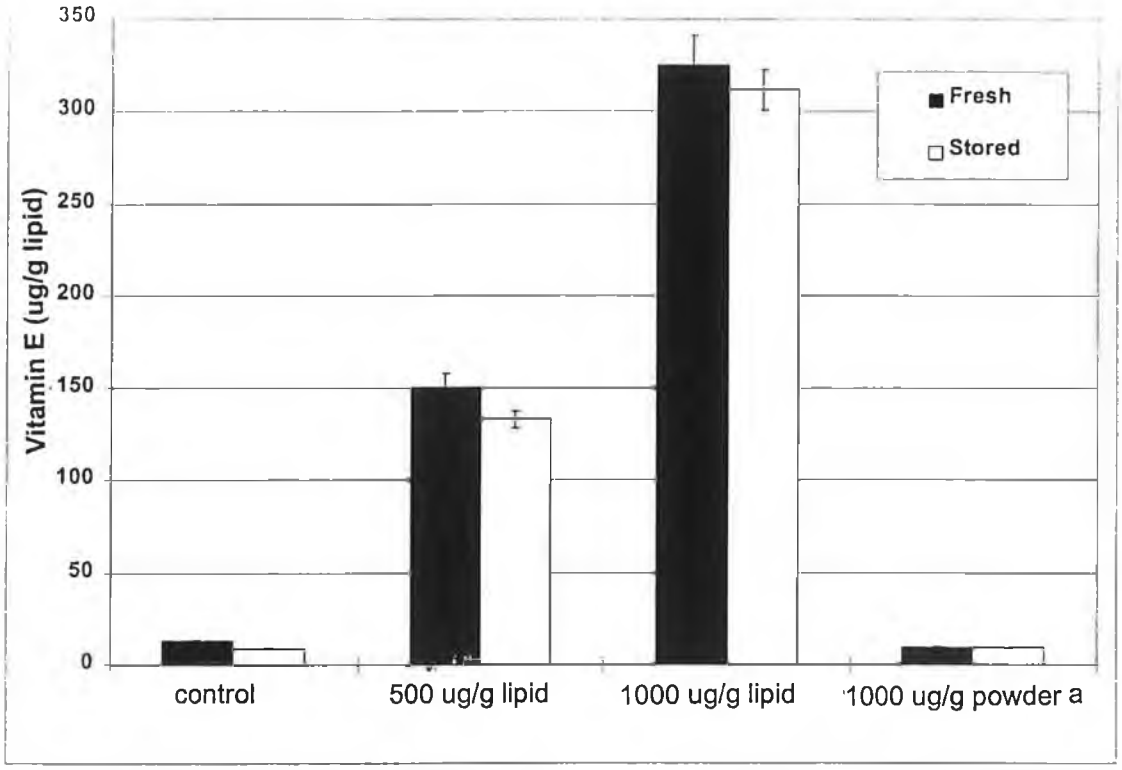

Fig. 4.6 Vitamin E levels in fresh (0 months) and stored (12 months) sachet-packed high-heat whole milk powders stored at 15° C. Data points refer to mean ± standard deviation (n= 4).

Levels of TBARS were not significantly different in fresh whole milk powder manufactured using either low or high doses of α-tocopherol supplemented milk compared to unsupplemented control (Fig. 4.7). However, a significant reduction in TBARS levels was seen in α-tocopherol supplemented sachet-packed whole milk powders stored at 15° C (Fig. 4.7) compared to unsupplemented controls. A 37.8 % reduction in TBARS was observed when 500 μg/g of α-tocopherol was added to milk prior to spray-drying and a similar reduction in TBARS (36.2 %) was observed in powders into which 1000 μg/g α-tocopherol had been incorporated prior to manufacture. An approximate reduction in TBARS of 12 % was observed in 500 μg/g lipid and 1000 lipid μg/g α-tocopherol supplemented sachet-packed whole milk powder stored at 30° C (Fig. 4.7). Hence, both levels of α-tocopherol investigated (500 μg/g lipid and 1000 μg/g lipid) produced similar reductions in TBARS development in sachet-packed powders during storage.

However the extent of lipid oxidation, as determined by TBARS, did appear to be dependent on α-tocopherol concentration in vacuum-packed powders stored at 15° C (Fig. 4.7). TBARS were reduced by 29.18 % and 52.63 % due to addition to the milk prior to spray-drying of 500 μg/g lipid and 1000 μg/g lipid doses of α-tocopherol, respectively. Similarly, in vacuum-packed powders stored at 30° C, the higher level of α-tocopherol (1000 μg/g lipid) produced a greater inhibitory effect than the 500 μg/g lipid dose on TBARS development during storage. When α-tocopherol was added to the milk powder after spray-drying, no protective effect was found, with levels of TBARS being similar to that of the control whole milk powder, to which no extra α-tocopherol was added.

Fig. 4.8 shows the levels of total COPs in fresh whole milk powders and in sachet and vacuum-packed powders stored at 15° C and 30° C, manufactured after addition of α-tocopherol (500 and 1000 μg/g lipid) to the milk prior to spray-drying. While the 1000 μg/g dose of α-tocopherol resulted in a 36.2 % reduction in TBARS in sachet-packed powders stored at 15° C (Fig. 4.7), this level of α-tocopherol had no

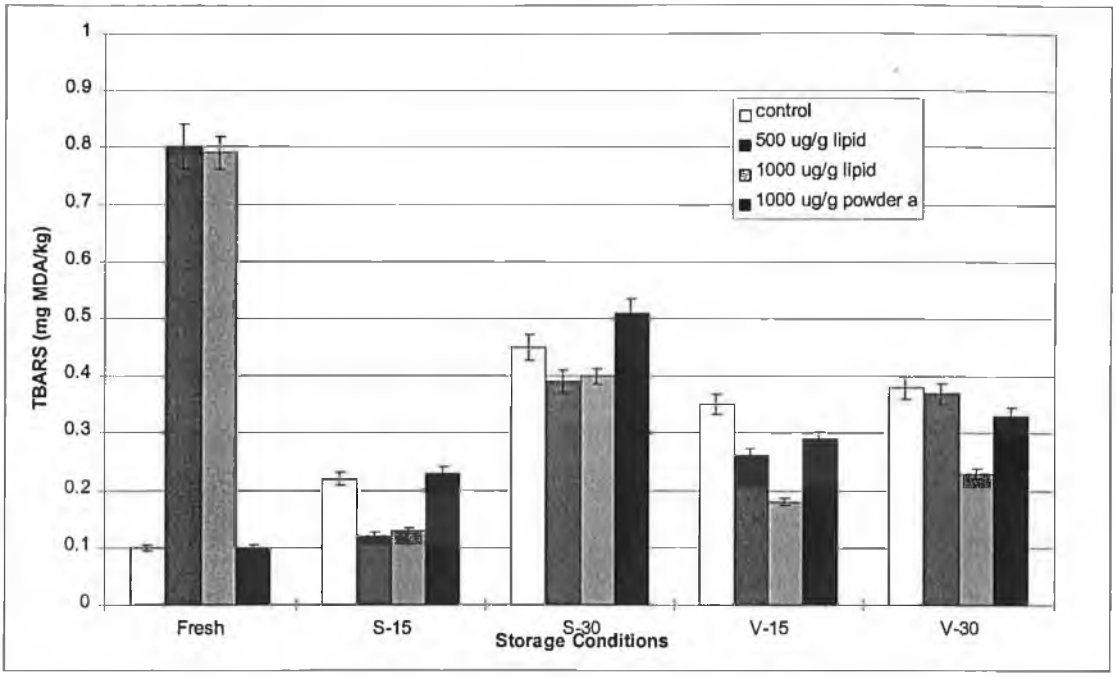

Fig. 4.7 Levels of TBARS in fresh and stored high-heat whole milk powders stored at 15° and 30° C for 12 months, sachet-packed (S) and vacuum-packed (V) after addition of α-tocopherol (500 μg/g lipid and 1000 μg/g lipid) to milk.
1000 μg/g powder [a] refers to data obtained when α-tocopherol was added to spray-dried powder. Data points refer to mean ± standard deviation (n= 4).

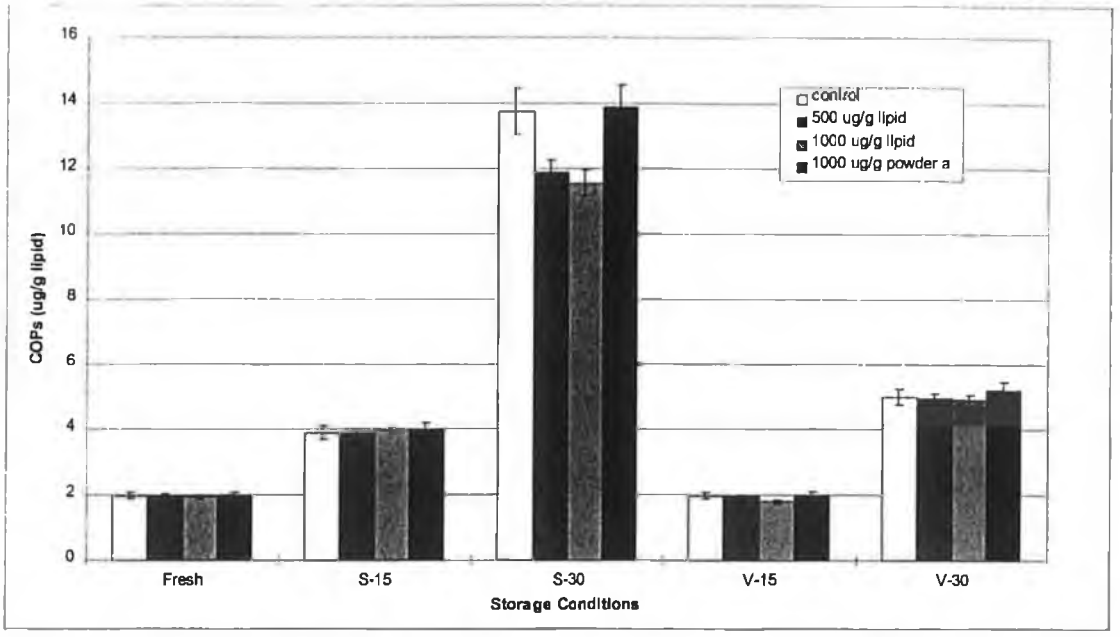

Fig. 4.8 Levels of COPs in fresh and stored high-heat whole milk powders stored at 15° C and 30° C for 12 months, sachet-packed (S) and vacuum-packed (V) after addition of α-tocopherol (500 μg/g lipid and 1000 μg/g lipid) to milk.
1000 μg/g powder[a] refers to data obtained when α-tocopherol was added to spray-dried powder. Data points refer to mean ± standard deviation (n= 4).

appreciable effect on COPs in sachet-packed powder stored at 15° C (Fig. 4.8). Levels of COPs found in control milk powder which were sachet-packed and stored at 15° C was 3.9 μg/g while levels of COPs in milk powders manufactured using milk to which was added 500 and 1000 μg/g α-tocopherol prior to spray-drying was 3.78 μg/g and 3.57 μg/g, respectively (Fig. 4.8). Storage at 30° C led to approximately 4-fold increases in COPs levels compared to storage at 15° C and addition of α-tocopherol (500 and 1000 μg/g) to the milk prior to spray-drying resulted in a 15-16 % reduction in COPs levels in these milk powders stored at 30° C. Thus, under similar packing and storage conditions, α-tocopherol was less effective in inhibition of COPs development than in the inhibition of lipid oxidation. These data demonstrate that mode of addition of α-tocopherol and storage conditions may influence the effectiveness of α-tocopherol in inhibiting oxidation of fatty acids in stored milk powders, and that milk fatty acids appear to be more sensitive to antioxidant activity of α-tocopherol than cholesterol oxidation.

α-Tocopherol appeared to have no effect on development of COPs in vacuum-packed powders stored at 15 or 30° C (Fig. 4.8), compared to control milk powders, to which no extra α-tocopherol was added. Although the level of total COPs increased when these vacuum-packed powders were stored at 30° C compared to 15° C, neither level of α-tocopherol in milk powder (500 or 1000 μg/g) resulted in any inhibitory effect on cholesterol oxidation.

In summary, addition of 500 and 1000 μg/g α-tocopherol to milk prior to spray-drying had no effect on COPs content in freshly produced powders which ranged from 1.65 - 1.73 μg/g. While the addition of 500 and 1000 μg/g α-tocopherol to milk prior to whole milk powder manufacture resulted in up to 53 % reduction in TBARS, no significant reduction in COPs development in these powders was observed.

4.6 DISCUSSION

The effects of pre-heat treatment of milk prior to evaporation and spray-drying on the physical, chemical, functional and organoleptic properties of whole milk powders were investigated.

Physical, chemical and functional characterisitics of manufactured whole milk powder

A single-stage spray-dryer was used in the manufacture of powders in the present study. One-stage drying is defined as the spray-drying process in which the product is dried to its final moisture content in the spray-drying chamber. This system allows control over moisture development at the exit from the drying chamber as well as influencing many other properties including insolubility index and bulk density. Organoleptic evaluation of fresh powders indicated an acceptable flavour which deteriorated in low-heat stored whole milk powders and which improved in high-heat powders upon storage (Table 4.1).

Moisture content, one of the most important physical properties of a powder, was not significantly affected by pre-heat treatments or storage conditions. The moisture contents of powders used in this study were all < 4 %. In addition, insolubility index was not significantly affected by pre-heat or storage conditions although low-heat powders did have lower insolubility indices (higher solubility) (0.1 ml) than high-heat powders (0.4 ml) and there was a trend towards an increase during storage at 15° C to 0.3 and 0.6 ml, respectively. Insolubility has been reported to increase if the storage temperature is high or if the moisture is low (Kiesker and Clarke, 1984; Kudo *et al.*, 1990). Although crystallisation of lactose (Saito and Taguchi, 1980) has been reported to be a possible cause for the increase in the insolubility index, Van Mil and Jans (1991) did not find any evidence of crystal formation. The term 'insolubility index' instead of 'solubility index' was introduced when the IDF modification of the original method was issued (Van Mil and Jans, 1991) and is determined as the volume of insoluble sediment remaining after reconstitution and centrifugation. It is often considered as one of the most important quality criteria of whole milk powders. The occurrence of insoluble sediment is undesirable, but to

some extent unavoidable. The main factor causing solubility defects is the particle temperature during the water removal process (Pisecky, 1986).

It has been reported that the storage stability of instant powder was maintained when the moisture content was < 4 % (Abbot and Waite, 1962) (Table 4.1). Van Mil and Jans (1991) studied the storage stability of whole milk powder having thee different moisture contents (2.4, 2.7 and 3.0 %) in tins and sachets at thee different temperatures (20, 27 and 35° C) over a thee year period. They observed that the insolubility index increased during the storage period and was more pronounced at the higher temperature or if the initial moisture content was high. The insolubility values found in this study were higher than those reported by Van Mil and Jans (1991), who reported values from 0.05-0.12 ml. This may have been due to differences in the pre-heating temperature; the powders of Van Mil and Jans (1991) being medium-heat powders, while low and high-heat powders were used in our study. In addition, there may have been differences in the inlet and outlet temperatures used in the spray-drying process. Both of these parameters affect the insolubility index of whole milk powders. Increased pre-heat temperatures and holding times have been shown to increase the viscosity of skim milk concentrate (Bloore and Boag, 1982). Furthermore, it has been demonstrated that increased viscosity of concentrate, independent of total solids can lead to increased insolubility index of resultant skim milk powder during the spray-drying process (Baldwin *et al.*, 1980). Therefore, the effect of pre-heating on whole milk powder solubility most likely arises from the effect of the pre-heat treatment on the concentrate viscosity.

The bulk density of the whole milk powders produced in the present study ranged from 0.51 to 0.56 g/ml. These values are similar to those previously reported by Van Mil and Jans (1991), who reported values from 0.53-0.57 g/ml. The bulk density of milk powders is economically, commercially and functionally an important property. When shipping powders over long distances, producers are interested in high bulk density to reduce shipping volume and to increase savings in packing.

The effect of pre-heat treatment on protein solubility of whole milk powder was determined by undenatured whey protein remaining in the powders after processing. The undenatured whey protein nitrogen index (WPNI) measures the proportion of undenatured protein remaining after a heat treatment. The low-heat powders had a mean WPNI value of 6.9 ± 0.9 mg undenatured whey protein/g powder while the high-heat powders had a mean WPNI of 1.2 ± 0.3 mg undenatured whey protein/g powder. As expected, high pre-heating temperatures (110° C) caused significantly ($p < 0.001$) more protein denaturation than low pre-heating temperature (75° C). These results were in agreement with previous data including Sanderson (1970) who reported values of 8.38 and 1.27 mg undenatured whey protein/g powder for low and high-heat powders, respectively. Similarly, Van Mil and Jans (1991) reported WPNI values of 1.6 mg undenatured whey protein/g powder for high-heat powders.

A slight decrease in WPNI values was observed after 12 months storage in the present study. Fresh low and high-heat whole milk powders contained 6.9 and 1.2 mg undenatured whey protein/g powder, respectively, which decreased to values of 6.6 and 1.1 mg undenatured whey protein/g, powder for corresponding stored powders respectively. This data indicating that the effect of storage on WPNI is negligible agrees with previous results (Baldwin & Ackland, 1991). Because the heat treatment classification is obtained indirectly by measuring the level of residual undenatured whey proteins, the usefulness of the undenatured WPN level as an index of heating history is limited by the extent of natural variation in the concentration of these proteins in raw milk. Whey protein was significantly higher ($p < 0.05$) in the standardised milk of the grass plus concentrate-fed herd S than that of the grass-fed herd, which may account for the higher WPNI in the resultant whole milk powder, but does not explain the seasonal effect. However, previous studies (Sanderson, 1970) have shown a seasonal effect on WPNI, which may in part explain our findings. Sanderson (1970) suggested that the seasonal variation in WPNI values may be due to the variation in individual whey protein components during the season, especially β-lactoglobulin and α-lactalbumin. Since the individual whey proteins were not determined in the present study, it is not possible to make such comparisons with the data of Sanderson (1970).

The average fat content of the powder was 26 %, which coincides with specific grading requirements for whole milk powders according to ADMI (1971) (Table 4.1). The raw milk was standardised to 3.2 % fat to produce a final fat content in the powder of 26 % according to Pearson's Square (Varnam and Sutherland, 1994). The oxidative stability, measured by PV and TBARS, deteriorated substantially in the stored powders. The chemical changes induced in milkfat by the process of manufacturing whole milk powders was further examined under different pre-heat, packing and storage conditions.

Effect of pre-heat treatment on oxidative stability during storage of whole milk powder

PV levels of fresh low-heat and high-heat powders in our study, which ranged from 0.40 - 0.43 mEq O_2/kg were high relative to the PV levels reported for anhydrous milkfat (Keogh and Higgins, 1982). The IDF standard for anhydrous milkfat (IDF 67A :1977) specifies a PV of less than 0.2 mEq O_2/kg by the ferric ammonium thiocyanate method. Stine *et al.* (1954) reported PVs of 0.15 - 0.36 mEq O_2/kg for fresh powders. The differences between the results of our study and others may be attributed to variations in processing technology and also in the methods used for PV assessment. The standardised IDF method was used in the present study, which differed from that employed by Stine *et al.* (1954) in that toluene replaced benzene in the fat extraction step. Numerous analytical methods exist for PV determination and the results of analysis depend upon the experimental conditions and upon the reducing agent used. Low-heat whole milk powders exhibited the highest levels of primary oxidation products assayed by PV.

Pre-heat temperature had no significant effect on secondary lipid oxidation products, in fresh milk powders, quantified by TBARS. The TBARS levels of whole milk powder samples obtained by low and high pre-heat treatment were 0.11 and 0.08 mg MDA/ kg, respectively. Stored low-heat whole milk powders, however, had significantly ($p < 0.01$) higher levels of TBARS than stored high-heat powders.

Chan (1992) reported values of 0.017 to 0.02 mg MDA/ kg in low-heat powders immediately after manufacture, which coincide with the results of the present study. Mettler (1973) indicated that a TBARS value of 0.008 was representative of freshly produced whole milk powder. High pre-heat treatment of milk has previously been shown to result in significantly less oxidation in whole milk powder than low pre-heat treatment, during subsequent storage (Boon, 1976; Tuohy and Kelly, 1981). In accordance with the results from the present study, Boon (1976) found that the highest level of off-flavour at any given storage time was observed in the powder which had received the low intensity heat treatment. High-heat treatment resulted in powders which developed oxidised flavour at about half the rate of the powders which had received a low pre-heat treatment. Tuohy and Kelly (1981) reported that a heat-treatment of 120° C resulted in a better storage stability than one of 80 or 90° C. Poulsen *et al.* (1971) stated that a heat-treatment of 95° C or above should be used for the manufacture of whole milk powder with good storage stability. One of the earliest studies on COPs in whole milk powder reported that after 12 months storage under ambient conditions, low-heat whole milk powder showed higher levels of COPs (17 μg/g lipid) than high-heat whole milk powder (9 μg/g lipid) (Nourooz-Zadeh and Appelqvist, 1988).

Freshly manufactured high-heat powders had significantly higher ($p < 0.05$) levels of COPs than low-heat powders (Fig. 4.4). The level of COPs detected in fresh low-heat whole milk powder manufactured in the present study was negligible (0 - 0.66 μg/g lipid). Previously, non-detectable levels of cholesterol oxides have been reported in fresh spray-dried milk powders, which were pre-heated at low and medium temperatures while levels of 2.8 μg/g lipid COPs were reported in fresh high-heat powders (Nourooz-Zadeh and Appelqvist, 1988). In our study, COPs levels in all powders stored for 12 months increased significantly ($p < 0.001$). As previously described for primary and secondary oxidation products, low-heat powders contained the highest levels of COPs after storage. Apart from cholestanetriol and 25-hydroxycholesterol, the high-heat powders used in this study contained similar cholesterol oxides to the low-heat powders, but the quantities recovered differed substantially between the two classes. The first detectable and

most prevalent oxide formed was 7-ketocholesterol, which confirms it's usefulness as a marker of cholesterol autoxidation (Maerker, 1987). Nourooz-Zadeh and Appelqvist (1988) and Chan *et al.* (1993) also reported that the predominant cholesterol oxide was 7-ketocholesterol. This oxide is of particular interest, from a biological point of view, because it is one of the products occurring in oxidatively modified low-density-lipoproteins (Addis and Warner, 1992), which play a role in the initiation of atherosclerosis and has been shown to be absorbed to the greatest extent from the intestine (Emanuel *et al.*, 1991).

The increased oxidative stability of high-heat powders relative to low-heat powders, as indicated by the reduced levels of PV, TBARS and COPs in high-heat powders after storage may be due to the increased levels of 'free' sulphydryl groups in these powders. High pre-heat treatment of milk increased 'free' sulphydryl groups ($p < 0.05$) in the resultant powders compared to low pre-heat treatment (Table 4.2). The observation that heat-treatment increased 'free' sulphydryl groups has been made previously (Harland *et al.*, 1952; Taylor and Richardson, 1980; Baldwin and Ackland, 1991). Taylor and Richardson (1980) have demonstrated that sulphydryl groups are responsible for only part of the anti-oxidant activity in milk. Exposure of reactive sulphydryl groups during pre-heating of milk has previously been observed to result in increased oxidative stability in the resultant whole milk powder by scavenging of free radicals (Harland *et al.*, 1952). Baldwin and Ackland (1991) also found that sulphydryl groups increased with pre-heating temperature. They found that the sulphydryl group measurement became 'saturated' at the higher pre-heat temperatures and that pre-heat holding-time had little effect. Increasing the temperature to 125° C and the hold-time to 240 sec did not increase the SH group levels found. Other workers (Kisza and Rotkiewicz, 1974) have reported that addition of heat-treated whey protein with a high content of sulphydryl groups improves the storage stability of whole milk powder. This is in accordance with Srinavasan and Singh (1974) who found that addition of sulphydryl groups to condensed milk products improved the storage stability. Ipsen and Hansen (1988) reported an inhibition of oxidation in whole milk powder with a high content of sulphydryl groups, but reported the inhibition to be the result of the total antioxidant

ability of the powder and not only due to the presence of sulphydryl groups. In the present study, while the level of 'free' sulphydryl groups increased as pre-heat treatment increased, the total sulphydryl groups decreased on heating. A similar observation has been previously reported (Taylor and Richardson, 1980) where skim milk which was heated for 30 min at 130° C showed greater antioxidant activity than raw milk. In addition, high-heat treated skim milk contained 48 % more reactive sulphydryl groups than raw milk, but 73 % fewer total sulphydryl groups (Taylor and Richardson, 1980). The exact reason for this phenomenon is not known.

Studies have shown that various Maillard products formed though the interaction of proteins and carbohydrates during high-heat-processing of foods exert antioxidant activity. Although Maillard reaction products were not measured in the present study, it is possible that their presence in high-heat powders may have contributed to the improved oxidative stability. The ability of Maillard reaction products to retard the development of rancidity in milk powders has been reported by a number of researchers (Wyott and Day, 1965; Erickson, 1982; Lingnert and Hall, 1986; Baldwin and Ackland, 1991).

Effect of packing on oxidative stability of whole milk powder

Vacuum-packing reduces the oxygen content of packed powder by both evacuation of the interstitial air and compression of the powder (Tuohy, 1984). Packing type had a marked effect on extent of lipid oxidation in the present study, where a significant reduction ($p < 0.05$) was observed in PV levels in vacuum-packed powders compared to sachet-packed powders (Fig. 4.2). In addition, a significant ($p < 0.01$) interaction existed between pre-heat temperature and packing conditions, i.e., the effect of pre-heat temperature on the oxidative stability of whole milk powder was more pronounced in sachet-packed powders than vacuum-packed powders. The present study also demonstrated that vacuum-packing resulted in significantly lower TBARS values ($p < 0.01$) and COPs levels ($p < 0.001$) compared with sachet-packing over the full experimental period. It has been previously reported that oxidative stability of lipid and cholesterol in whole milk powder can be

maintained for extended periods by exclusion of oxygen during subsequent storage (Chan *et al.*, 1993). However, although oxidation was reduced by vacuum-packing in the present study, a complete retardation of oxidation was not obtained. Residual oxygen may still cause lipid and cholesterol deterioration of milk powder in enclosed vacuum-packed systems and Tamsma *et al.* (1961) noted that extremely low oxygen concentrations (0.1 %) are necessary to prevent oxidative flavours in reconstituted foam spray-dried powder. The level of oxidation may have been reduced further if an inert gas such as nitrogen or carbon dioxide had been used to flush the powder prior to vacuum-packing. Lea *et al.* (1943) concluded that the development of oxidised flavours in spray-dried whole milk could be controlled by reducing the oxygen content in the free space of the container to 1 to 3 %, whereas Coulter and Jenness (1945) concluded that less than 1 % oxygen in the packing gas was necessary to prevent oxidation in whole milk powder. Full cream milk powder which had been packed under an inert gas, contained only traces of COPs (< 250 ng/g powder), even after storage of the unopened cans for 1 year (Rose-Sallin *et al.*, 1995). These authors also reported that the use of tin cans for packaging protected against cholesterol oxidation to a greater extent than aluminium foil bags, with COPs levels of 155 ng/g and 344 ng/g obtained for infant formula packed in tins and foil bags, respectively.

The variation in levels of lipid and cholesterol oxidation products between our study and those of others may to a large extent be due to the different methods for COPs quantification used, thereby making any direct comparisons between the data from different studies difficult. We used a modified version of the method of Sander *et al.* (1989) and Pie *et al.* (1990) for COPs quantification by GC and obtained similar COPs data to those reported by Rose-Sallin *et al.* (1995) for milk powder stored at ambient temperature for a 12-month period. Differences in methodologies are accounted for by the use of different extraction procedures, exclusion of a saponification step, variation in choice of internal standard, variation in choice of clean-up procedure and variations in the GC column used for COPs detection. Chan *et al.* (1993) and Sander *et al.* (1989) both used the Folch procedure for lipid extraction unlike Nourooz-Zadeh and Appelqvist (1988) who employed a

hexane/isopropanol extraction and saponification. Nourooz-Zadeh and Appelqvist (1988) used cholestane, Chan *et al.* (1993) 6-ketocholesterol and Rose-Sallin *et al.* (1995) and Sander *et al.* (1989) 19-hydroxycholesterol as the internal standard. Sander *et al.* (1989) did not employ a clean-up procedure, whereas others (Morgan and Armstrong, 1989; Chan *et al.*, 1993 and Rose-Sallin *et al.*, 1995) incorporated an SPE clean-up step, as in the present study. There is significant variation in the standard mixes used for total COPs quantification (Nourooz-Zadeh and Appelqvist, 1988; Morgan and Armstrong 1992; Sander *et al.*, 1989; Chan *et al.*, 1993; Rose-Sallin *et al.*, 1995) which further contributes to the variations reported in 'total' COPs values.

Effect of storage temperature on oxidative stability of whole milk powder

In the present study, higher storage temperatures (30° C) resulted in increased PV, TBARS (Fig. 4.2) and COPs levels (Fig. 4.4) in whole milk powder compared to the lower storage temperature (15° C). Chan *et al.* (1993) reported that TBARS and COPS values of powders stored at 40° C increased more rapidly (approximately 3-fold) than those stored at 20° C, while COPs levels were 12-fold higher at 40° C than 20° C. Huber *et al.* (1995) also reported that elevated storage temperature led to increased individual cholesterol oxide levels in spray-dried egg yolk. Lea *et al.* (1943) estimated an average temperature coefficient (Q_{10}) of 2.2 for milkfat oxidation in whole milk powder based on oxygen absorption, PV development and development of a tallowy flavour in powders stored at 37° C and 47° C. White *et al.* (1947) subsequently reported that there was a tendency for the Q_{10} to be lower for low temperature pre-heated samples than for high temperature pre-heated samples. White *et al.* (1947) found a Q_{10} of 1.6 for PV development in whole milk powder, measured over the temperature range 20-55° C, which is similar to those found by Tuohy (1987). Q_{10}, the average temperature coefficient, was not determined in this study, as only two temperatures were used, and a range of temperatures is needed to calculate Q_{10}. However, estimations of the rates of increase of PV and TBARS were obtained by comparing their values in samples stored at 30° C and 15° C. Average factors of 0.91 and 1.7 per 10° C were estimated for PV and TBARS development, respectively in sachet-packed powders stored over a 12 month period. The

magnitude of the increase in TBARS values illustrates the profound effect of storage temperature on lipid oxidation in whole milk powders.

Heat has been found to stimulate scission of cholesterol 7-hydroperoxides (Fontana *et al.*, 1993), and in our study both sachet and vacuum-packed low-heat and high-heat powders showed higher levels of COPs development when stored at 30° C compared to storage at 15° C (Fig. 4.4). The storage temperature effect on COPs levels was more pronounced when powders were sachet-packed than vacuum-packed indicating a positive interaction between storage temperature and packing ($p < 0.001$). This may have been due to the greater volume of headspace oxygen in sachet-packed powder, thus increasing the rate of lipid and cholesterol autoxidation. A significant interaction was also found between storage temperature and pre-heat temperatures ($p < 0.001$), i.e., the effect of pre-heat temperature was more pronounced at the higher storage temperature of 30° C.

The quantities of secondary cholesterol oxides increased at the higher storage temperature (Table 4.6). Secondary products of cholesterol are breakdown products of the primary oxidation products and hence are more highly oxidised. They have also been reported to be more cytotoxic and atherogenic than the primary oxidation products (Smith and Johnson, 1989). Because cholesterol-β-epoxide, 25-hydroxycholesterol and cholestanetriol are secondary oxidation products of cholesterol, it is apparent that the elevated temperature accelerated the oxidative process. In general, cholesterol-β-epoxide was present in greater quantities in low-heat powders stored at 30° C than cholesterol-α-epoxide. This can be explained by the greater thermodynamic stability of the equatorial over that of the axial conformation (Smith, 1981). The latter also indicated that the exposure of cholesterol to high temperatures for a relatively long period of time in the presence of oxygen may initiate the allylic free radical reaction at C_7. Following the formation of C_7 peroxy radicals, a series of free radical reactions take place which may result in the formation of stable oxidative products. Storage of samples at 30° C can lead to a more intense oxidative degradation of cholesterol than storage at 15° C.

Chan *et al.* (1993) also reported significantly higher secondary oxidation products of cholesterol at 40° C compared to 20° C.

The rate of increase of PV was greater than of TBARS during the first 6 months of storage. PV values reached a maximum at 6 months and decreased during the subsequent 6 months under all conditions of storage (Fig. 4.2). That peroxide values are at best only a transitory measurement of lipid oxidation (Tuohy, 1987) is seen from this study. The highest level of peroxides were found after 6 months storage, in the sachet-packed low-heat powders stored at 30° C, after which they declined. Similar observations have been made by Lea *et al.* (1943) who reported that the PVs of spray-dried whole milk powders stored at 37° C reached a maximum between fifty and one hundred days which coincided with the point at which all the free oxygen in the storage can had been consumed. At this point, peroxide formation ceased and the PV decreased again as peroxide break-down proceeded. Similar patterns of peroxide value development were reported by Pyenson and Tracy (1946) and White *et al.* (1947). Van Mil and Jans (1991) and Tuohy (1987) found that peroxide values reached a maximum in whole milk powders after 18 and 12 months, respectively. Van Mil and Jans (1991) concluded that due to the complex mechanism of autoxidation reactions, products can be formed after an extended time that do not contribute to the PV assay result but can lead to a recovery of the flavour score. This may be due the Maillard browning (a reaction between lysine groups of proteins and carbonyl groups of lactose) that occurs during pre-heating and/or storage, which has a sweet taste and can lead to an improvement in flavour.

Correlation of COPs with lipid oxidation products

Previously, secondary oxidation products (TBARS) have been positively correlated with COPs production during storage in cooked pork (Monahan *et al.*, 1992) and in whole milk powder (Chan *et al.*, 1993). Recent observations by Nielsen *et al.* (1996), however, suggests that COPs production in dairy spreads is linked to the early stages of lipid oxidation. The difference may be due to a number of reasons. Firstly, Nielsen *et al.* (1996) studied dairy spreads whose fat content is greater than

that of whole milk powder. In addition, the two different products makes it difficult to make direct comparisons. Also, the TBA acid distillation assay of King (1962), employed by Nielsen *et al.* (1996) differed from that of others (Monahan *et al.*, 1992; Chan *et al.*, 1993) making direct comparison of TBARS levels in these studies difficult.

Effect of α-tocopherol addition on oxidative stability of whole milk powder

Since the transfer of vitamin E from feed to milk by the dairy cow is relatively ineffective, with only 2 % secreted into the milk (King, 1968), it was of interest to investigate the effect of direct addition to milk prior to processing. α-Tocopherol has been reported to be a more effective antioxidant than BHA (Rankin and Pike, 1993), and hence its antioxidative properties were investigated in the present study. Also, it has been found that irrespective of the concentrations of the tocopherols in feedstuffs, only α-tocopherol is found in significant concentrations in the blood and tissues of pigs and cattle and therefore, only α-tocopherol was considered as biologically important (Rice and Kennedy, 1986). Loss of α-tocopherol as a result of whole milk powder manufacture was greater than 60 % (Fig. 4.6). Dziezak (1986) also reported that tocopherols can be generally lost during refining, deodorising and processing of food because of their heat lability. Spontaneous milk oxidation was found to be inversely proportional to the α-tocopherol content (Dunkley *et al.*, 1967) who also noted seasonal variations in α-tocopherol concentration in milk. The fact the α-tocopherol level is higher than that found in the whole milk powders produced in the present study may be due to dietary differences or seasonal variations between the studies.

A protective effect against lipid oxidation was found due to addition of α-tocopherol to the milk prior to spray-drying. The level of oxidation was significantly higher in sachet-packed powders stored at 30° C compared to storage at 15° C. However, α-tocopherol incorporation did not offer a similar degree of protection against cholesterol oxidation as observed against lipid oxidation in our study (Fig. 4.8). Similar observations have been previously made, where no significant difference

was observed in COPs development in spray-dried egg powders due to incorporation of antioxidants (TBHQ and oleoresin rosemary), although a slight reduction was observed (Lai *et al.*, 1995b). Similarly, Wahle *et al.* (1993) found that α-tocopherol levels of approximately 500 μg/g resulted in some inhibition of cholesterol oxidation, though not statistically significant. In the present study, α-tocopherol was incorporated into two of the whole milk powder manufacturing trials (in duplicate), and hence only trends can be observed.

When α-tocopherol was added in a powder form to whole milk powder after the spray-drying process, the protective effect against lipid oxidation was not observed. The vitamin E concentration in the supplemented powder to which 1000 μg/g was added was only 11 μg/g lipid, compared to 9 μg/g lipid in the control powder to which no extra α-tocopherol was added. This may have been due to localised mixing of the α-tocopherol and poor inadequate mixing of the entire powder sample. The α-tocopherol added prior to spray-drying was emulsified (using glyceryl monostearate) in the concentrated milk prior to homogenisation and spray-drying. This was a pre-requisite, as α-tocopherol is immiscible with water. Emulsified tocopherol has previously been shown to be effective at concentrations of 25-100 μg/g lipid while non- emulsified forms of α-tocopherol were essentially ineffective in preventing oxidised flavour (King, 1968). Control of oxidised flavour by direct addition of emulsified tocopherol to milk required less than 1/100 of the amount of vitamin E required by supplementing the diet (King, 1968). Although antioxidants did not completely retard lipid and cholesterol oxidation, the formation of oxidation products was reduced to some extent and further work needs to be undertaken to ascertain the optimum levels required for reduction of both lipid and cholesterol oxidation in whole milk powders.

Effect of processing, packing and storage on organoleptic properties of whole milk powder

Little data is available on the relationship between TBARS value and flavour acceptability of whole milk powder. Biggs and Bryant (1953) reported TBARS values for a series of powders with flavour descriptions ranging from 'fresh' to

'very oxidised'. 'Fresh' powders had a TBA value of 0.04 mg MDA/kg, a 'slightly oxidised' powder had a TBA value of 0.087 mg MDA/kg, the powders described as 'oxidised' ranged in TBA value from 0.16 mg MDA/kg, to 0.24 mg MDA/kg, and two powders described as 'very oxidised' had TBA values of 0.50 mg MDA/kg and 5.2 mg MDA/kg. However, the procedure used by Biggs and Byrant was based on the method of Dunkley and Jennings (1951) while the method of Tarladgis *et al.* (1960), was used in the present study and therefore TBA values are not directly comparable between the two sets of data. Tuohy (1987) concluded that the flavour of powders with a TBA value $\leq$ 0.19 mg MDA/kg would be regarded as stable and powders with a TBA value of $\geq$ 0.19 mg MDA/kg would be classified as spoiled. Based on Tuohy's findings (1987), we can conclude that all powders manufactured in the present study were of acceptable flavour when stored at 15° C for up to 4 months and at 30° C up to 2 months (Fig. 4.2). However, on sensory analysis, high-heat vacuum packed powders of milk from both herds which were stored for 12 months were of acceptable flavour, which may indicate a variation between sensory analysis and the TBA assay (Table 4.4). Ward (1985) reported that in some instances, good correlations between the TBA value and sensory results can be obtained but reliable and reproducible correlations are not achieved generally (Chan, 1993). Tuohy (1987) concluded that although the relationship between TBA development and sensory perception of flavour acceptability may be empirical, it is clear that powders with a low TBA value have a high degree of flavour acceptability. Thus, high-heat pre-heat treatment, vacuum-packing and ambient storage conditions resulted in reduced levels of lipid oxidation and the most acceptable flavour score for stored whole milk powder.

4.7 CONCLUSIONS

In whole milk powders stored up to 12 months, primary oxidation products were dominant during the first 6 months while secondary oxidation dominated during the subsequent 6 months storage. Milk powders from the grass plus concentrate-fed herd S resulted in less protein denaturation in low-heat powders and higher levels of 'free' sulphydryl groups than those from grass-fed which received grass only. This may have contributed to the lower levels of lipid and cholesterol oxidation found in

fresh and stored powders from herd S. The elevated antioxidant status (α-tocopherol and sulphydryl groups) in whole milk powder from cows of herd S undoubtedly also contributed to reduced lipid and cholesterol oxidation. High pre-heat temperatures resulted in higher levels of COPs in fresh whole milk powders, but led to a more stable product during subsequent storage. Both lipid and cholesterol oxidation increased under all storage conditions investigated, but these undesirable ageing effects could be further reduced by exclusion of oxygen and reduced storage temperatures. Seventy percent of added α-tocopherol was lost due to the whole milk powder manufacturing process. While the increased level of antioxidant inhibited lipid oxidation during storage of these powders at ambient temperatures, cholesterol oxidation was not inhibited to the same extent. This may be due to inability of the α-tocopherol added in the present study to inhibit cholesterol oxidation in addition to lipid oxidation.

CHAPTER 5

INVESTIGATION OF TOXICITY OF CHOLESTEROL OXIDATION PRODUCTS IN AN *IN VITRO* MODEL SYSTEM

5.1 INTRODUCTION

Several of the oxidation products of cholesterol have adverse biological activities and have been implicated in many diseases including cancer (Black and Douglas, 1973; Petrakis *et al.*,1981; Smith and Johnson, 1989) and atherosclerosis (Imai *et al.*, 1976; Peng *et al.*, 1979; Jacobson, 1987; Addis and Warner, 1992). Cholesterol oxides exhibit cytotoxicity toward a wide variety of cells leading to angiotoxic and atherogenic effects (Imai *et al.*, 1976). They have been shown to alter prostaglandin synthesis (Weksler *et al.*, 1977) and stimulate platelet aggregation (Ross, 1986). Evidence of cytotoxicity have been obtained from indices of cytotoxicity from studies on cultured fibroblasts (Baranowski *et al.*, 1982), smooth muscle cells (Hughes *et al.*, 1994), endothelial cells (Hu *et al.*, 1991), monocytes and macrophages (Baranowski *et al.*, 1982), heart cells (Mersel *et al.*, 1987), skin cells (Williams *et al.*, 1987) and granulocytic progenitor cells of bone marrow (Hoffman *et al.*, 1981). These studies showed an increased rate of cell death within hours of exposure to COPs, inhibition of cell growth, rounding up of cells and detachment from the surface of cell culture flasks, decreased incorporation of thymidine into DNA, suppression of cellular cholesterol biosynthesis, the appearance of intracellular lipid droplets and accumulation of cholesteryl esters (Peng *et al.*, 1992 a). In general, cytotoxicity of individual cholesterol oxides appears to vary depending on the type of cell line exposed and whether cells are in a proliferating or a quiescent state. The influence of duration of exposure to cholesterol oxides may vary depending on the nature of the compound and on the dose.

The exact mechanism of cholesterol oxide toxicity is unclear. Since many cholesterol oxides have been shown to be potent inhibitors of cholesterol biosynthesis (Kandutch and Chan, 1975; Erickson *et al.*, 1977), a possible mechanism of action may be that they deplete the cellular cholesterol available for membrane synthesis, thereby affecting the molar ratio of cholesterol to phospholipid in membranes (Peng and Taylor, 1984; Peng *et al.*, 1992 b). Furthermore, their similar molecular structure to cholesterol facilitates their direct insertion into cell membranes. The latter may be a separate mechanism for cytotoxicity since studies on mature red blood cells that do not synthesise cholesterol have shown that

cholesterol oxides can greatly alter cell surface shape as a result of their direct insertion into membranes (Streuli *et al.*,1981; Santillan *et al.*, 1982). Analysis of subcellular distribution profiles of incorporated cholesterol oxides show that some COPs such as 25-hydroxycholesterol are evenly distributed thoughout the mitochondrial and microsomal fractions, the plasma membrane-enriched fraction and the nuclear pellets, while others such as cholestanetriol are localised preferentially in plasma membranes (Peng *et al.*, 1985). These modifications in cholesterol content by COPs have been shown to be extensive enough to alter membrane fluidity and affect a number of membrane functions, including properties of membrane-bound enzymes (De Pierre and Karnovsky, 1973), receptor-mediated endocytosis and cell proliferation (Peng and Morin, 1992).

A number of mechanisms exist for defence of tissues against free radical damage (Halliwell and Chirico, 1993; Rice-Evans, 1994) (Fig 6.1). Protection against the effects of COPs may be afforded by the antioxidant status of cells *in vivo*. The adult human with a daily energy requirement of 10,000 kJ (2,500 kcal) consumes approximately 660 g oxygen/day. It is estimated that approximately 90-95 % of that oxygen is converted by mitochondrial respiration to harmless water, whereas the remaining 5-10 % of oxygen undergoes univalent and divalent reduction yielding reactive species, such as superoxide radicals, hydrogen peroxide and hydroxyl radicals (Esterbauer, 1993). Extrapolated to the average human lifespan of approximately 70 years, the human body consumes approximately 17,000 kg of oxygen, and this results in the concomitant production of 800-1700 kg of oxygen radicals (Esterbauer, 1993). In view of the potential damage that may be caused by free radicals and hydroperoxides, and the possible direct effect of various oxidants on the cellular components, it is important that the cell contains antioxidant defence systems (Chow, 1979). All organisms suffer exposure to radicals: hydroxyl radicals are generated by homolytic fission of O-H bonds in water (Duthie, 1991; Halliwell, 1994) while approximately 1-4 % of the total oxygen uptake by mitochondria is used in superoxide formation (Duthie, 1991). Although superoxide is not itself particularly reactive, in the presence of copper, iron and other initiators, a metal

catalysed Haber-Weiss reaction may occur, resulting in the formation of the highly reactive hydroxyl radicals.

Living organisms have evolved antioxidant defences to remove excess superoxides and hydrogen peroxide (Halliwell, 1994; Rice-Evans and Gopinathan, 1995) (Fig 6.1). The first line of defence against such radicals is the enzyme superoxide dismutase (SOD), which dismutes superoxide anion radical to hydrogen peroxide. Because SOD enzymes generate hydrogen peroxide, they work in concert with hydrogen peroxide-removing enzymes, such as catalase (CAT) and glutathione peroxidase (GPx) (Halliwell, 1994; Lawlor and O'Brien, 1994; Descampiaux *et al.*, 1996). Catalase reduces hydrogen peroxide to oxygen and water (Chow, 1979). The selenium containing enzyme, glutathione peroxidase is one of the most important hydrogen peroxide-removing enzymes in mammalian cells (Chow, 1979). A selenocysteine residue, essential for enzyme activity, is present at the active site of glutathione peroxidase (Halliwell and Chirico, 1993). GPx remove hydrogen peroxide by using it to oxidise reduced glutathione (GSH). Glutathione reductase regenerates GSH from GSSG, with NADPH as a source of reducing power (Halliwell, 1994).

α-Tocopherol is a free radical scavenger that has been reported to protect against oxidative stress *in vitro* (Lawlor and O'Brien, 1994) and inhibit lipid peroxidation (Burton and Ingold, 1981) thus maintaining cell membrane integrity (Diplock, 1983). In addition α-tocopherol has been reported to protect against toxicity of COPs *in vitro* (Hughes *et al.*, 1994; Wilson *et al.*, 1995) at concentrations ranging from 1-2 μM. There is increasing evidence that in certain circumstances the protective systems may be overwhelmed and that cell damage by oxygen radicals and lipid peroxidation play a crucial and causative role in the pathogenesis of several chonic and acute diseases such as cancer (Vavavi *et al.*, 1994), inflammation, shock, liver injury, rheumatoid arthitis, ageing and atherosclerosis (Esterbauer, 1993). As mentioned earlier, the COPs most commonly found in food are the hydroxycholesterols and epoxycholesterols, 7-ketocholesterol, cholestanetriol and

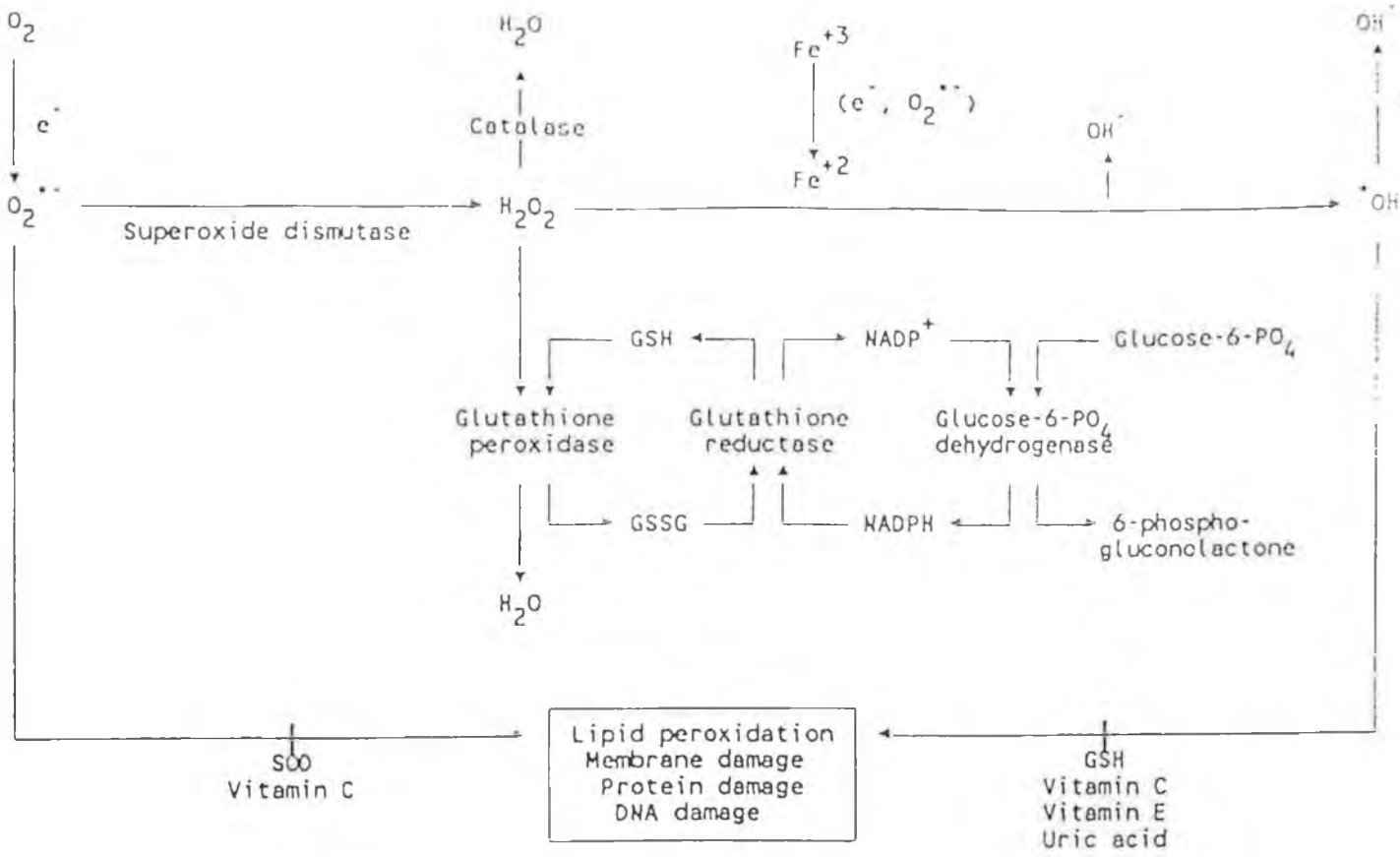

Fig. 5.1 The antioxidant defence system (Kukucka and Misra, 1993).

25-hydroxycholesterol (Nourooz-Zadeh and Appelqvist, 1988; Paniangavait *et al.*, 1995).

The previous chapters indicated that cholesterol of whole milk powders destined for dietary use can readily autoxidise in the presence of air at high storage temperatures and that formation can be significantly influenced by the animal feed quality of the milk producers, extent of pre-heating during manufacture and the presence of oxygen during storage. Cholesterol oxides can also be endogenously produced *in vivo*, either via free radical-induced chain reactions, such as lipid peroxidation (Smith, 1981; 1982) or by enzyme-catalysed reactions, notably though microsomal and mitochondrial cytochome P_{450} systems (Smith and Johnson, 1989). That cholesterol oxides themselves might influence cholesterol metabolism and/or cell morphology and function in cells other than liver and vascular tissues needs further exploration. By influencing cell and tissue viability they could eventually be implicated in the aetiology of disease processes other than atherosclerosis and cancer.

This chapter reports on the effects of cholesterol oxides on a cell model known to express significant steroidogenic potential. Porcine granulosa cells are found lining the ovarian follicle and as the ovary increases in size during the menstrous or oestrous cycle, the granulosa cells multiply (Baker, 1982). Fig. 5.2 shows the stages of follicular growth. At birth, each oocyte is surrounded by a single layer of flattened epithelial-derived granulosa cells and the combined structure is called a primordial follicle (Fig. 5.2). Most primordial follicles remain in the arrested state, which may last until puberty or even menopause, if they are not selected for further differentiation and development (Hadley, 1992). With the initiation of the reproductive cycle, the process of follicular growth culminates in ovulation (Baker, 1982). Within the embryonic ovary, the primordial follicles begin the reproduction division of mitosis (primary follicles), resulting in an increase in size of the oocyte and a change in shape of the granulosa cells from flat to cuboidal (secondary follicles) (Fig. 6.2) (Baker, 1982; Hadley, 1992). Although a number of primary follicles may be selected for further development into secondary follicles during each

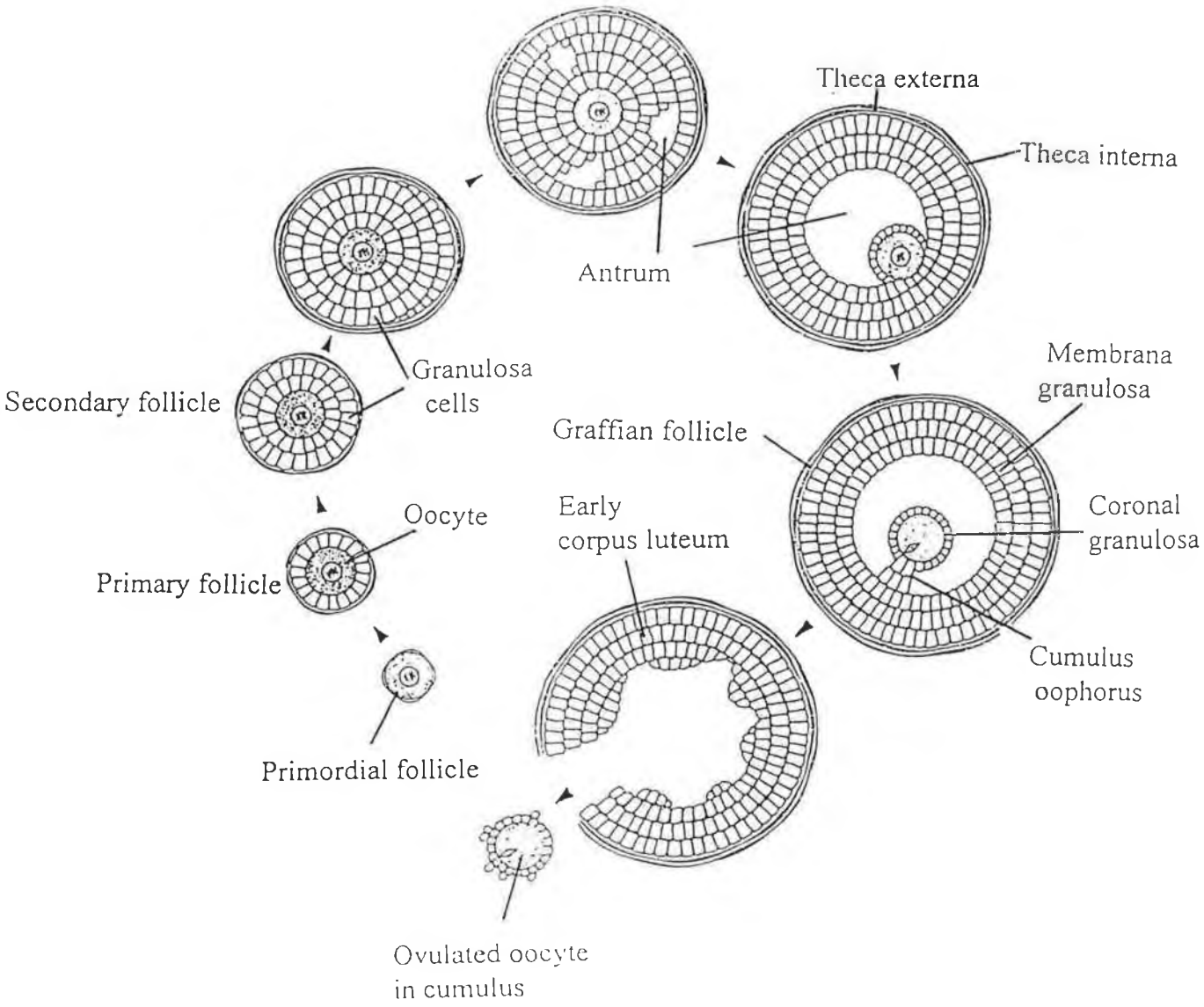

Fig. 5.2 Stages of follicular growth (Baker, 1982).

menstrual cycle, only one is usually destined to develop into a Graffian follicle. The others by some unknown process become atretic (Hadley, 1992).The granulosa cells of follicles destined for maturation continue to increase in number. At the same time, interstitial tissue adjacent to the follicle arrange around the follicle to form the theca. The cells adjacent to the follicle, the theca interna, are surrounded by an additional outer layer of interstitial cells, the theca externa. Continued proliferation of granulosa cells and the incorporation of surrounding interstitial cells into the theca are accompanied by the accumulation of fluid in spaces or clefts within the granulosa cells. As the follicle enlarges, a single large vesicle or antrum is formed. Those granulosa cells that are adjacent to the ovum comprise the 'coronal granulosa' cells; those granulosa cells that remain in contact with the surrounding theca cells are called the 'membrana granulosa'. A bridge of granulosa cells connects those cells adjacent to the ovum with the 'membrana granulosa' and is called the 'cumulus oophorus'. The mature follicle at this stage of development is called the 'Graafian follicle'. During ovulation, the 'cumulus oophorus' is severed, and the ovum is ejected. After rupture of the Graffian follicle, the granulosa cells start to fill up, accumulate large quantities of cholesterol and this process leads to the formation of the 'corpus luteum'.

The control of ovarian steroid synthesis and secretion is a complex process involving the interaction of the granulosa and theca cells, as well as a co-ordinated sequence of actions by the pituitary gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH) (Erickson, 1983). Gonadal tissues are actively steroidogenic using cholesterol as a precursor. Granulosa cells in the ovaries are responsible for the production of C18 oestrogens, with the Leydig cells of the testes being the equivalent cell type in the male (Kukucka and Misra, 1993). The C18 oestrogens are produced via an aromatisation conversion of C19 androgens, delivered to the granulosa cells from the theca cells where they function in ovum maturation (Hadley, 1992). The aromatase system consists of thee enzymes located on the endoplasmic reticulum; a 19-hydroxylase enzyme, a 19-hydroxysteroid dehydrogenase and a 10, 19-lyase enzyme (Vance, 1993). The 19-hydroxylase enzyme, a mixed function oxidase having cytochome P_{450} at the active site, is found

in the mitochondria and endoplasmic reticulum of steroid producing tissues (Vance, 1993). Studies in recent years have shown that the activities of cytochome P_{450} hydroxylase and lyase enzymes in testes Leydig cells are decreased in the presence of O_2-generated free radicals (Kukucka and Misra, 1993), even though the mechanisms of oxygen toxicity have not been investigated in detail.

As discussed earlier, during normal oxidative metabolism, cells continually convert molecular O_2 into superoxide radical and hydrogen peroxide and under normal conditions, most cells contain antioxidant defences for detoxifying and eliminating these reactive species of oxygen (Fig. 5.1). If, however, the normal defences are weak or there is an overwhelming production of free radicals, more toxic reactive oxygen species are formed including the potent hydroxyl radical and singlet oxygen, which are capable of evoking numerous pathological phenomena including protein alteration and cell membrane damage (Clare *et al.*, 1995).

Abnormal aromatase activity may therefore adversely affect oestrogen biosynthesis and thereby affect ovum maturation. In view of the active P_{450} enzyme system in granulosa cells and it's likely dependence on the oxidative status of cells, it was of interest to investigate whether metabolism in ovarian granulosa cells might be adversely influenced by cholesterol oxides via damage to cellular and subcellular endoplasmic reticular membranes and/or altered antioxidant defence system functioning.

Granulosa cells have many advantages for culture studies, being relatively simple to obtain in reasonably pure form, in large numbers and at known stages of maturation (Hall, 1994). In female mammals, all germ cells are formed in the ovary before birth, many of which become atretic, so that by puberty only half the initial number of oocytes remain. Therefore, any agent that damages the oocyte will accelerate the depletion of oocyte number, thereby reducing fertility (Garside and McKibbin, 1987). The survival of a species therefore depends on the integrity of it's reproductive system and the possible toxic effects of oxysterols on the reproductive system is an important health issue.

This chapter investigates the oxidative status of granulosa cells when exposed to some COPs previously found to exist in whole milk powders. This entailed setting up an *in vitro* model system in which activities of antioxidant defence enzymes were determined in cells exposed to a range of COPs in the presence and absence of α-tocopherol. The concentration of each COP (2.5 μM in the well which is equivalent to 1μg/ml) that was chosen for this study was based on the evidence derived from a human feeding study carried out by Emanuel *et al.* (1991). It was established from that study that the levels of total COPs in plasma ranged between 200-1600 μg/dl (average 617 μg/dl) after ingestion of a test meal of powdered eggs containing 230 μg/g lipid COPs. Thus, the levels of COPs found in sachet-packed whole milk powder stored at 30 °C for 12 months (37.17 ± 7.54 μg/g lipid) is equivalent to 1 μg/g lipid in plasma assuming a linear dose response of each COP and similar absorption of each COP.

5.2 AIMS

To investigate the toxicity of a number of COPs found in whole milk powder.

5.3 OBJECTIVES

The objectives of this study were:

- To investigate the effect of COPs on cell growth using primary cultures of porcine ovarian granulosa cells.
- To determine the effect of COPs on development of cellular lipid peroxidation.
- To determine the effects of COPs on the antioxidative defence enzyme systems.
- To determine the effect of α-tocopherol when administered simultaneously with COPs on the antioxidant defence enzyme systems.

5.4 EXPERIMENTAL

5.4.1 Reagents

New-born calf serum (NCS), non-essential amino acids, penicillin-streptomycin, L-glutamine, trypan blue, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cytochome C, glutathione reductase, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADP), reduced glutathione, pyruvic acid, superoxide dismutase, tert-butyl hydrogen peroxide, xanthine, xanthine oxidase and malondialdehyde (MDA) were all purchased form Sigma Chemical Co. (Poole, Dorset, UK). DMEM (Dulbecco's modified Eagle medium) and medium 199 without phenol red were purchased from GIBCO (Paisley, Scotland).

5.4.2 Recovery of granulosa cells from porcine ovaries

Porcine ovaries were taken from freshly slaughtered pigs at a local abbatoir (Dennys, Tralee, Co. Kerry) and placed in ice-cold PBS (8 g NaCl, 0.2 g KCl, 2.89 g Na_2HPO_4 and 0.02 g KH_2PO_4/l). Excess tissue was removed from the ovaries, which were then washed in ice-cold PBS. Granulosa cells were collected by aspirating all follicles using a syringe and 21-gauge needle and centrifuged at 600 g for 5 min at 35° C. Cells were counted using a haemocytometer and were seeded at a final concentration of 2 x 10^5 /ml in complete DMEM containing 10 % NCS, 1 % penicillin-streptomycin and glutamine and cultured for 24 h at 37° C in an atmosphere containing 95 % O_2 and 5 % CO_2 in 24 well plates.

5.4.3 Cell Treatment

Twenty-four well plates were used for the bioassays, with random number tables used to assign treatments to wells. The treatments were as follows: control cells received 20 μl absolute ethanol, α-tocopherol controls received 10 μl absolute ethanol and 10 μl 208 μM α-tocopherol, while cells treated with COPs received 20 μl 248 μM 25-hydroxycholesterol, cholestanetriol, 7-ketocholesterol, cholesterol-α-epoxide or cholesterol alone (made up in ethanol). Cells treated with COPs and α-tocopherol simultaneously received 10 μl of 208 μM α-tocopherol and either 10 μl 498 μM 25-hydroxycholesterol or cholestanetriol dissolved in ethanol. The final

volume in the well was 2 ml and the final concentrations of α-tocopherol and COPs in the well were approximately 1.0 and 2.5 μM, respectively.

5.4.4 Cell Viability

5.4.4.1 Measurement of trypan blue exclusion by cells

Granulosa cell monolayers were dissociated in the culture wells using (0.25 w/v) trypsin/EDTA solution (100 mM EDTA in PBS, pH 7.4). The culture medium in each well was first removed by transfer pipette and discarded. Into each well, 0.5 ml of pre-warmed PBS (37° C) was added to wash out cell debris and to remove any remaining serum proteins which could impair trypsin action. The PBS was also removed by transfer pipette and discarded. Trypsin/EDTA solution (0.5 ml) was added to each well. The culture plates were then incubated at 37° C in the CO_2 incubator until all of the cells had become detached from the well floor. To inhibit trypsin activity, 0.5 ml complete DMEM (containing NCS) was added to each well. The contents of each well were repeatedly aspirated with a sterile pasteur pipette to obtain an even suspension of cells. An aliquot of the cell suspension was mixed with an equal volume of trypan blue and the cells observed under a Canon inverted microscope and counted using a haemocytometer. Using trypan blue stain, dead cells were stained blue. Results were expressed as % viability.

5.4.4.2 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

Cell viability was determined using the MTT assay (Mosmann, 1983): the culture media in each well was first discarded and the cells washed with 2 mls PBS. 200 μl of 10 % MTT (in M199 medium) was added and incubated at 37° C for 4 h. The medium was carefully discarded, taking care not to disrupt the formazan crystals. The crystals were dissolved in 2 ml isopropanol, the absorbance read at 570 and 690 nm and the difference calculated and expressed as % of control cells.

5.4.5 Antioxidant Defence Bioassays

5.4.5.1 Superoxide Dismutase (SOD) Assay

Total superoxide dismutase (SOD) activity in porcine ovarian granulosa cells was determined by the method of Galleano *et al.* (1994). This assay depends on SOD ability to inhibit cytochome C reduction mediated by $O_2^{\cdot}$ generated from the xanthine oxidase system (Lawlor and O'Brien, 1994). Cells were sonicated at 4° C for 2 min prior to assay. The assay was performed at 25° C in 2.5 ml 0.05 M potassium phosphate buffer, pH 7.8 to which was added 0.3 ml 50 µM xanthine oxidase and 0.2 ml 20 µM cytochome C in a cuvette and the initial absorbance monitored at 550 nm for 1 min. 100 µl of the cell extract was then added, the change in absorbance/min was recorded and results were expressed as activity units/mg protein. One unit of SOD activity is defined as the amount of SOD required to inhibit rate of cytochome C reduction by 50 %. The level of protein in the cell extract was determined and the results expressed as units/mg protein.

5.4.5.2 Catalase (CAT) Assay

Catalase (CAT) activity was measured using the method of Baudhuin *et al.* (1964). Cells were sonicated at 4° C for 2 min prior to assay and diluted 1:4 with 1% Triton X-100. The assay was performed at 25° C in 2.9 ml 0.01 M potassium phosphate buffer, pH 7.0 in a quartz cuvette to which was added 100 µl 0. 3 M H_2O_2, and the initial absorbance monitored at 240 nm. 10 µl of the cell extract was then added to the cuvette and the absorbance monitored at 240 nm over 30 sec. The amount of H_2O_2 reduced was determined by the Beer Lambert Law (for H_2O_2 $\varepsilon = 40\ M^{-1}\ cm^{-1}$). One Bergmeyer unit is equivalent to the amount of CAT required to decompose 1.0 µmol H_2O_2 /min/ml at pH 7.0 at 25 ° C. Results were expressed as Bergmeyer units/mg protein. Protein was determined according to the method of Bradford (1976).

5.4.5.3 Glutathione Peroxidase (GPx) Assay

GPx activity was measured according to the method of Guenzler *et al.* (1974). Reduced glutathione is oxidised by GPx and this reaction is coupled with the conversion of NADPH to NADP by glutathione reductase (Lawlor and O'Brien,

1994). The following reagents were added to a spectrophotometric cuvette: 1.63 ml 0.01 M potassium phosphate buffer, pH 7.7 containing 0.001 M EDTA, 100 µl 10 mM H_2O_2, 50 µl N_3Na, 30 µl 10 mM NADPH, 50 µl GSH reductase and 100 µl 100 mM GSH. The absorbance was monitored at 340 nm to determine the baseline. This was then repeated after adding 50 µl cell sample and the NADPH consumption rate recorded. The difference in NADPH consumption rate before and after addition of cell sample was calculated using the Beer Lambert Law (ε = 6.2 mM^{-1} cm^{-1}). One unit of GPx activity is defined as 1 µmol of GSH oxidised/min = 1 µmol NADPH oxidised/min. The GPx activity was expressed as units/mg protein. Protein was determined according to the method of Bradford (1976).

5.4.6 Measurement of lipid oxidation in cells using TBARS assay

The level of lipid peroxidation in cells was determined by the method outlined by Esterbauer and Cheeseman (1990). A 10 mM stock solution of malondialdehyde (MDA) was prepared using malondialdehyde bismethylacetal in 1 % sulphuric acid. This was left at room temperature for 2 h to achieve complete hydrolysis and was then used in the preparation of standards in the range 0.1- 100 µmol. Cell sample was sonicated on ice for 2 min and 1 ml of the cell extract was mixed with 2 ml of ice-cold 10 % (w/v) TCA to precipitate protein. The precipitate was pelleted by centrifugation at 1000 g and 1 ml supernatant reacted with an equal volume of 0.67 % w/v TBA in a boiling water-bath for 10 min. After boiling, all samples were left to cool and the absorbance was read at 532 nm. The actual concentration of MDA was calculated based on an ε value of 153,000 M^{-1} cm^{-1}. Results were expressed as nmol MDA/mg protein. Protein was determined according to the method of Bradford (1976).

5.4.7 Determination of total cellular protein

Protein was determined according to the Bradford assay (Bradford, 1976). Medium was discarded from wells and cells washed twice with 1 ml PBS. Cells were then sonicated for 3 min in 1 ml PBS, and 100 µl cell sample added to 5 ml Bradford reagent (0.01 % Coomassie Brilliant Blue, 4.7 % ethanol and 8.5 % phosphoric acid), allowed stand for 2 min and absorbance monitored at 595 nm. A standard

curve (0 - 100 μg/ml) was constructed using bovine serum albumin and results expressed as mg protein/ml.

5.4.8 Statistical Methods

Student t-tests were used for determining statistical significance of toxicity effects of COPs using Sigmastat (Jandel Scientific Software, USA).

5.5 RESULTS

5.5.1 Porcine ovarian granulosa cell growth curve

Fig. 5.3 shows a typical growth curve for porcine ovarian granulosa cells *in vitro*. The viable cell count was ascertained using the trypan blue exclusion test as outlined in Chapter 5, section 3.4.1. The growth curve indicates an initial 16.5 % mortality over the first 24 h of culture before the onset of an exponential phase of cell division. Analysis of the growth curve indicated that 24 h after plating was a suitable time for initiation of experiments involving exposure of these cells to COPs (Fig. 5.3) as this indicated the start of the exponential phase of growth.

5.5.2 Investigation of toxicity of COPs on porcine ovarian granulosa cells

Fig. 5.4 shows the effects of exposure to 2.5 μM cholesterol, triol, cholesterol-α-epoxide, 7-ketocholesterol and 25-hydroxycholesterol for 24 h on granulosa cell viability. Cholesterol and 7-ketocholesterol (2.5 μM) had no inhibitory effect on granulosa cell viability following 24 h exposure with reductions in cell viability of only 4 %. However, significant inhibition occurred in the presence of 2.5 μM 25-hydroxycholesterol, cholestanetriol ($p < 0.001$) and cholesterol-α-epoxide ($p < 0.01$) where cell viability was reduced by 66 %, 48 % and 44 %, respectively, following exposure of granulosa cells for 24 h.

5.5.3 Effect of extended incubation time with COPs on porcine ovarian granulosa cells

Fig. 5.5 shows a time course bioassay of exposure to 2.5 μM cholestanetriol and 25-hydroxycholesterol on cell viability after 24, 48 and 72 h incubation. Cell viability was significantly ($p < 0.01$) reduced at all times of exposure to COPs investigated though no significant difference was found between the different time periods for both oxides. Exposure of granulosa cells to cholestanetriol resulted in cell number reductions in the range 42 - 52 % over 72 h, while exposure to 25-hydroxycholesterol resulted in reductions of 66 %, 71% and 46 % in cell viability after 24 h, 48 h and 72 h exposure, respectively. An increase in viability of 25 %, though not significant, was observed when 25-

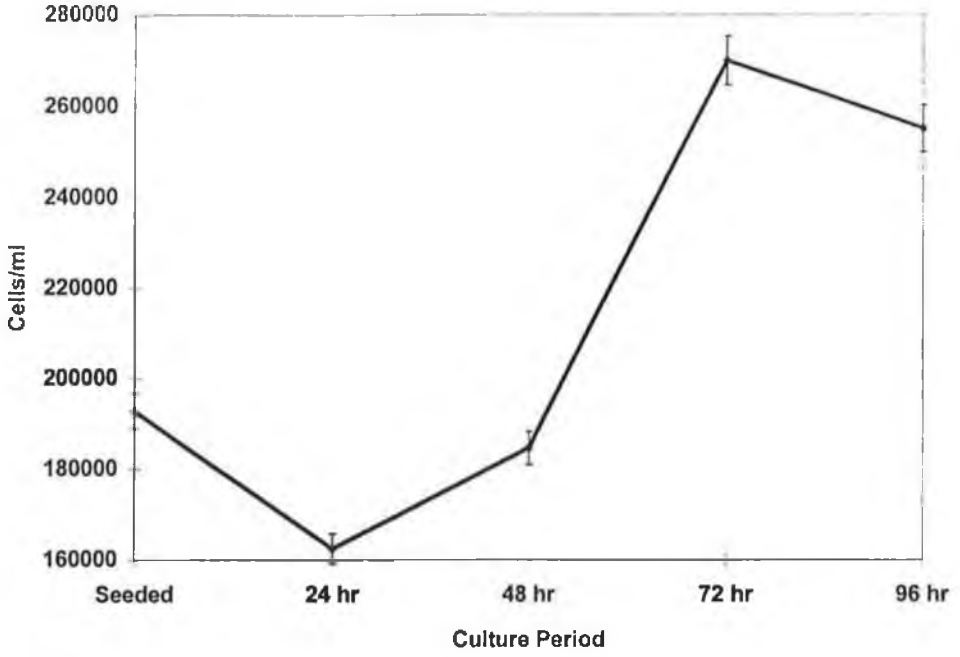

Fig. 5.3 Growth curve of porcine ovarian granulosa cells. Data points refer to mean ± standard deviation.

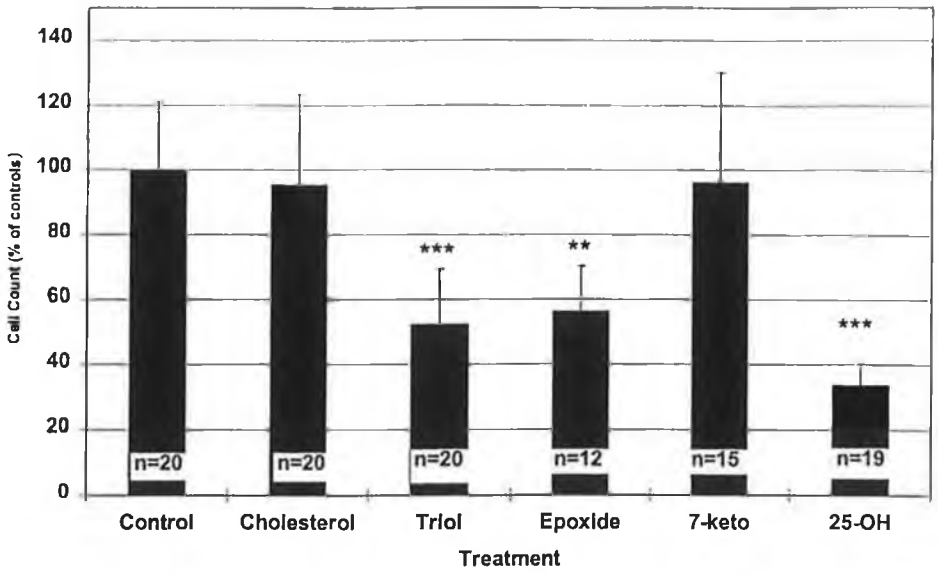

Fig. 5.4 Porcine ovarian granulosa cells were incubated with 2.5 μM cholesterol, cholestanetriol, cholesterol-α-epoxide, 7-ketocholesterol and 25-hydroxycholesterol and incubated for 24 h. Triol: Cholestanetriol; 7-keto: 7-Ketocholesterol; 25-OH: 25-Hydroxycholesterol; Epoxide: Cholesterol-α-epoxide. Viable cell count was then carried out using trypan blue exclusion dye. ** ($p < 0.01$), *** ($p < 0.001$) relative to control. Error bars refer to standard deviation.

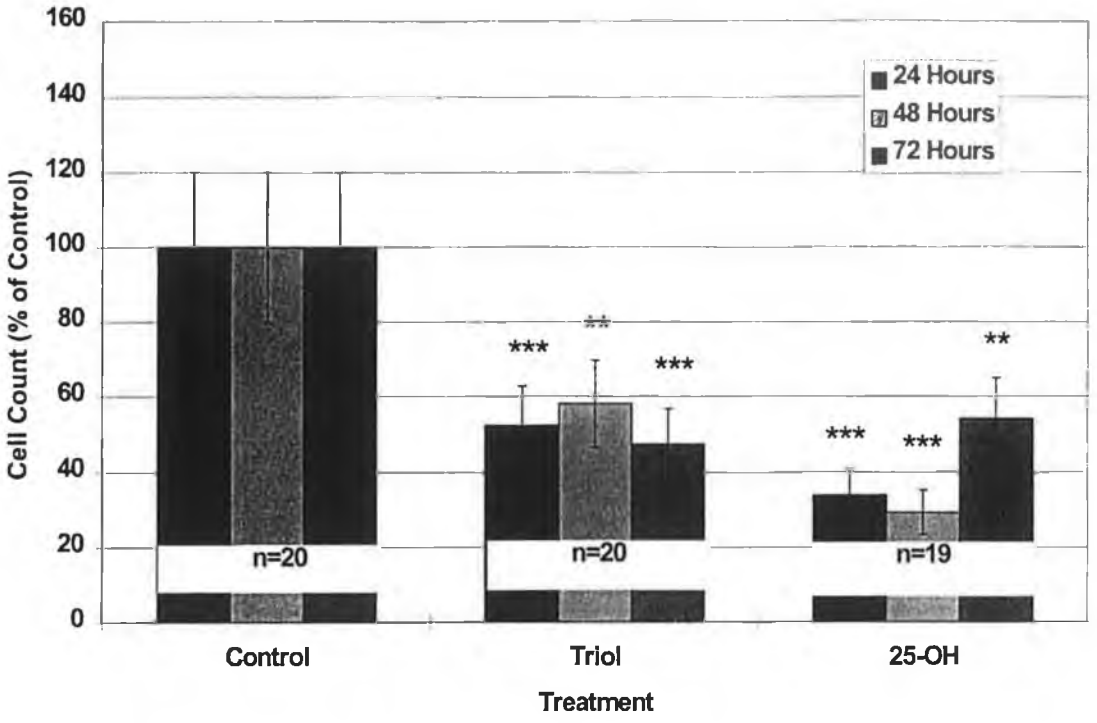

Fig. 5.5. Time course bioassay of 2.5 μM cholestanetriol and 25-hydroxycholesterol in porcine ovarian granulosa cells incubated for 24, 48 and 72 h. Triol: Cholestanetriol; 25- OH: 25-Hydroxycholesterol. Cell counts were then carried out as a measure of cell viability using trypan blue exclusion dye. ** ($p < 0.01$), *** ($p < 0.001$) relative to control. Error bars refer to standard deviation.

hydroxycholesterol was incubated for 72 h, indicating that the cells may have 'recovered' or adapted to the presence of 25-hydroxycholesterol after 72 h incubation. Since no significant difference in cell viability was observed between the thee incubation periods investigated, the 24 h incubation period was employed for all further bioassays in this study.

5.5.4 Effect of 25-hydroxycholesterol and cholestanetriol on cell number and viability

Fig. 5.6 shows the effect of incubation of granulosa cells with 2.5 μM cholestanetriol and 2.5 μM 25-hydroxycholesterol for 24 h on cell viability, using both the viable cell count (Chapter 5, section 4.4.1) and MTT assay (Chapter 5, section 4.4.2) as indicators of viability. The results indicate that when cells were exposed to either cholestanetriol or 25-hydroxycholesterol for 24 h, both cell viability and mitochondrial integrity were significantly ($p < 0.001$) reduced relative to untreated controls. When cells were exposed to 2.5 μM cholestanetriol, mitochondrial integrity were reduced by 26 %. Similarly, when cells were exposed to 2.5 μM 25-hydroxycholesterol, mitochondrial integrity was reduced by 38 %. In contrast, a similar concentration of cholesterol had little effect on mitochondrial integrity (11 % reduction in mitochondrial integrity).

5.5.5 Effect of α-tocopherol addition on response to COPs administration

Fig. 5.7 shows the effects of incorporation of α-tocopherol simultaneously with 25-hydroxycholesterol and cholestanetriol on cell viability and mitochondrial integrity. When α-tocopherol (1.0 μM) was incubated with control cells for 24 h, it had negligble effect on cell viability or mitochondrial integrity. However, when α-tocopherol was incorporated simultaneously with cholestanetriol for 24 h, cell viability and mitochondrial integrity were 1.4-fold and 1.2-fold greater than in the absence of α-tocopherol (1.0 μM). Thus, toxicity was reduced by 20 % and 16 %, as measured by trypan blue exclusion and MTT assays, respectively. When α-tocopherol (1.0 μM) was incorporated simultaneously with 25-hydroxycholesterol cell viability and mitochondrial integrity were 1.8-fold and 1.6-fold greater than in

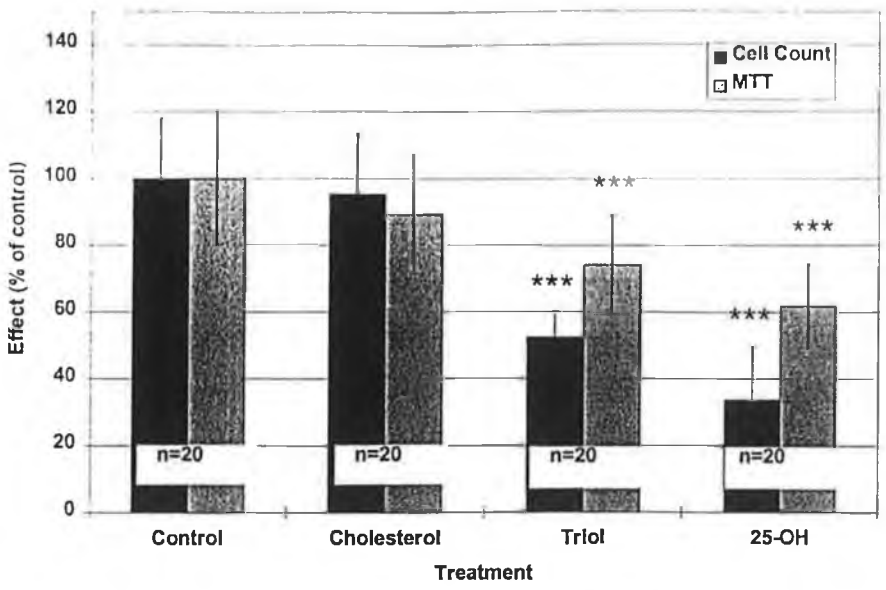

Fig. 5.6 Effect of 2.5 μM cholesterol, cholestanetriol and 25-hydroxcholesterol on cell viability and mitochondrial integrity in porcine ovarian granulosa cells after 24 h incubation. Triol: Cholestanetriol; 25- OH: 25-Hydroxycholesterol. Cell viability was determined using trypan blue exclusion dye and mitochondrial release was determined using the MTT assay. *** ($p < 0.001$) relative to control. Error bars refer to standard deviation.

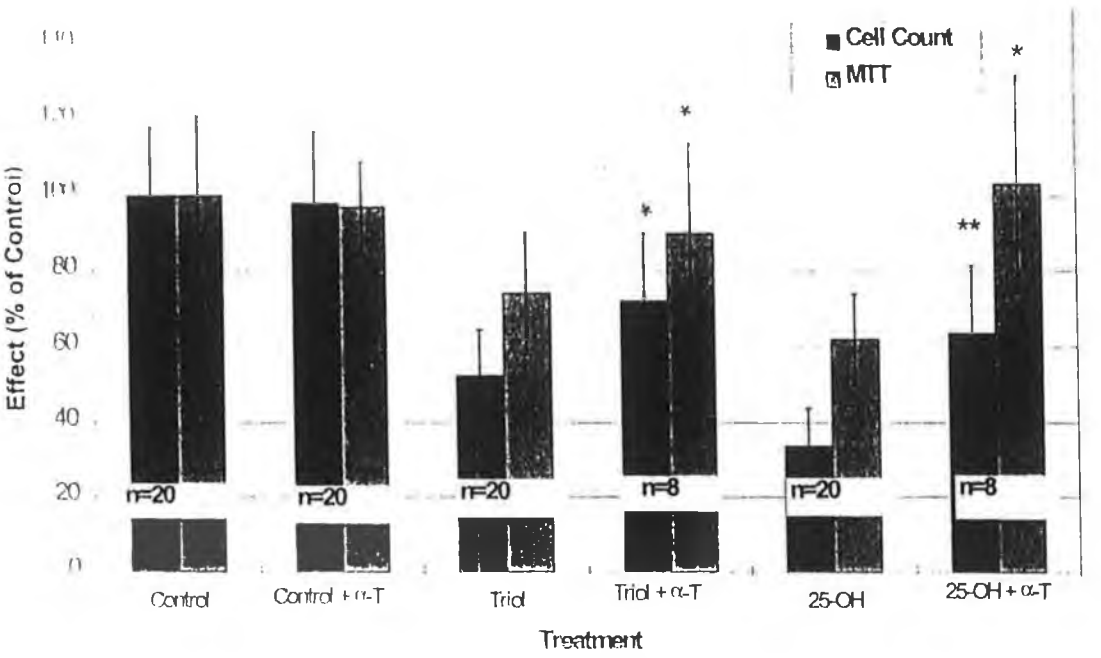

Fig. 5.7 Effect of simultaneous α-tocopherol (1 μM) addition with 2.5 μM cholestanetriol and 25-hydroxycholestrol on cell viability and mitochondrial integrity in porcine ovarian granulosa cells. Triol: Cholestanetriol; 25- OH: 25-Hydroxycholesterol; α-T: α-Tocopherol. Cell viability was ascertained using trypan blue and mitochondrial integrity was determined using the MTT assay. * ($p < 0.05$), ** ($p < 0.01$) relative to 25-OH. Error bars refer to standard deviation.

the absence of α-tocopherol indicating that α-tocopherol had a protective effect against the toxicity of COPs following 24 h exposure. Thus, toxicity was reduced by 30 % and 42 %, as measured by trypan blue exclusion and MTT assays, respectively.

5.5.6 Effect of COPs on lipid peroxidation in porcine ovarian granulosa cells

Fig. 5.8 shows the effect of administration of 2.5 μM cholestanetriol and 25-hydroxycholesterol in the presence and absence of 1.0 μM α-tocopherol on lipid peroxidation as determined by TBARS. Control cells showed a level of TBARS equivalent to 125 nmol MDA/ mg protein. Incubation with cholestanetriol and 25-hydroxycholesterol alone showed no significant effect on TBARS levels following 24 h exposure, compared to controls, being 100 and 121 nmol MDA/ mg protein, respectively. However, when α-tocopherol was incubated in combination with 25-hydroxycholesterol, a reduction of 35 % in TBARS was observed, though not statistically significant. A negligible reduction was seen on α-tocopherol incubation with cholestanetriol, though not significant.

5.5.7 Effect of COPs on SOD, CAT and GPx activities in porcine ovarian granulosa cells

Porcine ovarian granulosa cells (2×10^5 / ml) were incubated with 2.5 μM cholestanetriol and 25-hydroxycholesterol for 24 h to determine their effect on the activities of the antioxidant enzymes SOD (Fig. 5.9), CAT (Fig. 5.10) and GPx (Fig. 5.11) in porcine ovarian granulosa cells. A significant increase in SOD activity was found when these cells were incubated with 2.5 μM cholestanetriol (0.91 activity units/mg protein) ($p < 0.05$) and 25-hydroxycholesterol (2.13 activity units/mg protein) ($p < 0.01$) relative to control cells (0.47 activity units/mg protein) (Fig. 5.9). Simultaneous addition of α-tocopherol had no effect on SOD activity in control cells (0.47 activity units/mg protein). When α-tocopherol was administered simultaneously with 2.5 μM cholestanetriol for 24 h, SOD activity was significantly ($P < 0.001$) reduced to that found in control cells (0.58 activity units/mg protein). SOD activity of 25-hydroxycholesterol-treated cells was 4-fold higher than untreated

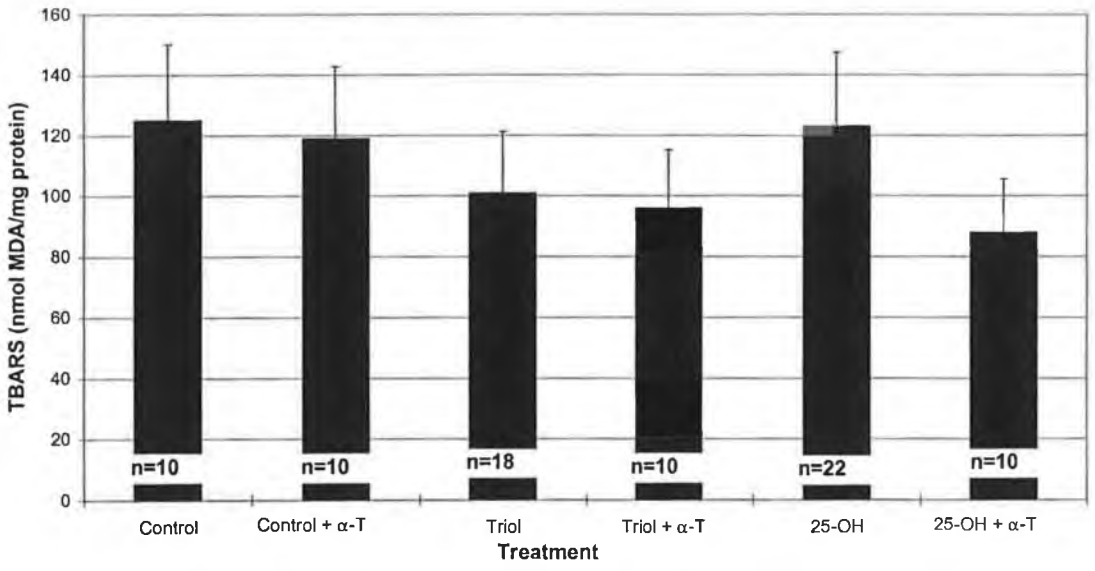

Fig. 5.8 Effects of 2.5 μM cholestanetriol and 25-hydroxycholesterol, in the presence and absence of 1.0 μM α-tocopherol on lipid peroxidation, as determined by TBARS, in porcine ovarian granulosa cells. Triol: Cholestanetriol; 25- OH: 25-Hydroxycholesterol; α-T: α-Tocopherol. Error bars refer to standard deviation.

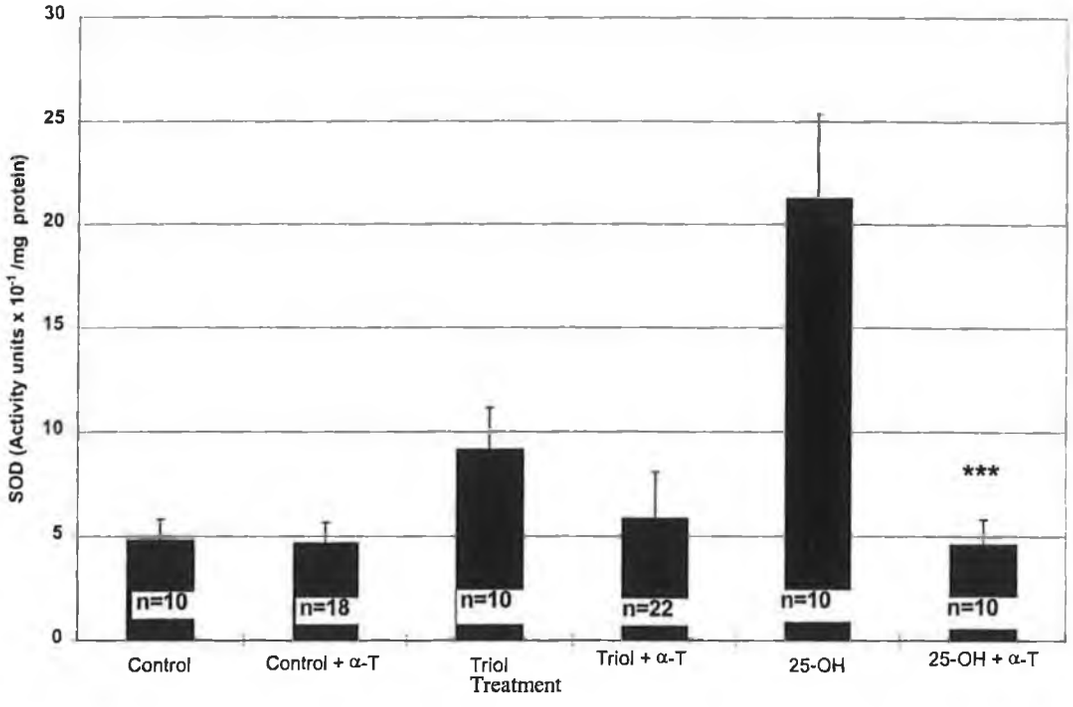

Fig. 5.9 Effects of 2.5 μM cholestanetriol and 25-hydroxycholesterol, in the presence and absence of 1.0 μM α-tocopherol on SOD activity after 24 h incubation.

Triol: Cholestanetriol; 25- OH: 25-Hydroxycholesterol; α-T: α-Tocopherol.

*** ($p < 0.001$) relative to 25-hydroxycholesterol.

** ($p < 0.01$) relative to control.

* ($p < 0.05$) relative to control.

Error bars refer to standard deviation.

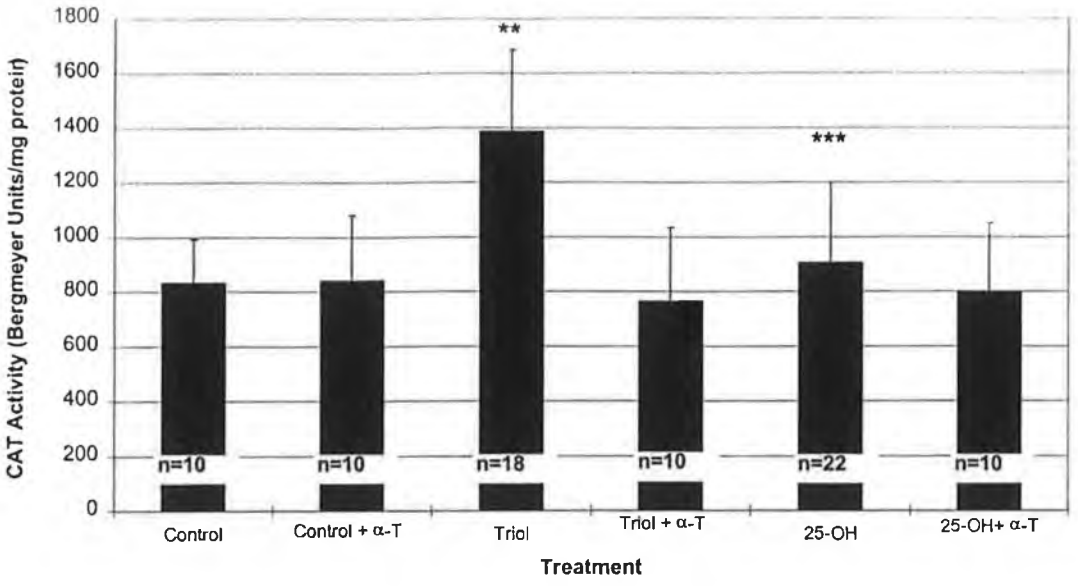

Fig. 5.10 Effects of 2.5 μM cholestanetriol and 25-hydroxycholesterol in the presence and absence of 1.0 μM α-tocopherol on CAT activity after 24 h incubation. Triol: Cholestanetriol; 25- OH: 25-Hydroxycholesterol; α-T: α-Tocopherol.
** ($p < 0.01$), *** ($p < 0.001$) relative to control. Error bars refer to standard deviation.

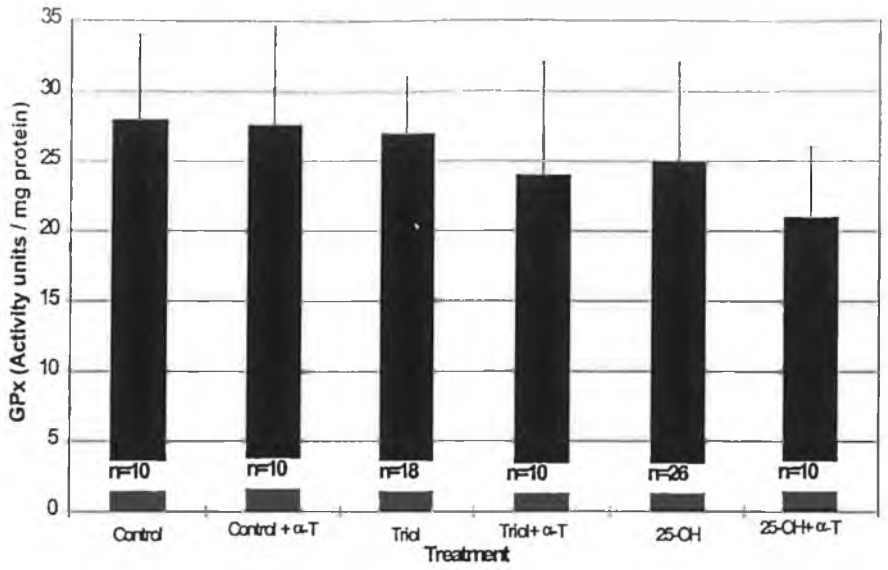

Fig. 5.11 Effects of 2.5 μM cholestanetriol and 25-hydroxycholesterol in the presence and absence of 1.0 μM α-tocopherol on GPx activity after 24 h incubation. Triol: Cholestanetriol; 25- OH: 25-Hydroxycholesterol; α-T: α-Tocopherol. Error bars refer to standard deviation.

controls, which was returned to control levels by simultaneous incorporation of 1.0 μM α-tocopherol with 2.5 μM 25-hydroxycholesterol.

Fig. 5.10 shows the effect on CAT activity of exposure of granulosa cells to 2.5 μM cholestanetriol and 25-hydroxycholesterol. Control cells had a level of CAT activity equivalent to 833 Bergmeyer units/mg protein. Approximately 1.5-fold and 1.2-fold increases in CAT activity were observed when cells were treated with cholestanetriol (1388 Bergmeyer units/mg protein ($p < 0.01$) and 25-hydroxycholesterol (906 Bergmeyer units/mg protein) ($p < 0.001$) relative to the control (Fig. 5.12). α-Tocopherol had no effect on CAT activity in control cells. When α-tocopherol was incorporated simultaneously with cholestanetriol and 25-hydroxycholesterol, CAT activity was reduced to levels similar to those of control cells.

Fig. 5.11 shows the effect on GPx activity of exposure of granulosa cells to 2.5 μM cholestanetriol and 25-hydroxycholesterol. Control cells had GPx activity equivalent to 27 units/mg protein. Cells treated with 2.5μM cholestanetriol and 25-hydroxycholesterol for 24 h had GPx activities similar to that of control cells. α-Tocopherol had no effect on GPx activity in control cells. Reductions of 11 % and 28 % in GPx activity were observed when cells were incubated with 2.5 μM cholestanetriol and 25-hydroxycholesterol in the presence of 1.0 μM α-tocopherol, respectively, compared to incubation with the cholesterol oxides alone. These reductions were not, however, statistically significant.

5.6 DISCUSSION

In the present study, porcine ovarian granulosa cells were investigated as a model system in which to study the effects of COPs. Other model systems have included Chinese Hamster Ovary cells (CHO) (Esterman *et al.*, 1983) and porcine aortic smooth muscle cells (SMC) (Hughes *et al.*, 1994). The latter have been used as a model system in which to study the toxicity of COPs (Peng *et al.*, 1978; Hughes *et al.*, 1994) at concentrations ranging from 1 μg/ml (Naseem and Heald, 1987) to 100 μg/ml (Peng *et al.*, 1985). 25-hydroxycholesterol and cholestanetriol have been reported to be toxic *in vitro* by a number of investigators (Smith and Johnson, 1989; Mahfouz *et al.,* 1995). Peng and Taylor (1984) incubated cultured aortic smooth muscle cells with a number of COPs at levels up to 100 times higher (100 μg/ml) than in the present study and after 24 h incubation found that 25-hydroxycholesterol and cholestanetriol were the most toxic. They assessed cell viability by morphology aberrations; viable cells were spindle shaped with distinct nuclei and cytoplasm and dying cells were small, dark round bodies with no nuclear or cytoplasmic detail. A more recent study (Mahfouz *et al.,* 1995) has shown that cholestanetriol (10 μg/ml) is capable of displacing cholesterol from cell membranes of rat-liver microsomes. CHO cells are a well-established model for the study of sterol synthesis (Sinensky *et al.*, 1980; Esterman *et al.*, 1983), and hence have been used to investigate the effect of COPs incubation on HMG-CoA reductase. Inhibition of HMG-CoA reductase has been reported in CHO cells on incubation with COPs at concentrations ranging from 0.1 μg/ml (Chin and Chang, 1981) to 1.5 μg/ml (Sinensky and Mueller, 1981; Esterman *et al.*, 1983).

Effect of COPs on cell viability

Cholestanetriol, 25-hydroxycholesterol and cholesterol-α-epoxide were found to significantly ($p < 0.01$) inhibit cell viability while 7-ketocholesterol and cholesterol had no inhibitory effect on cell viability (Fig. 5.4). The two most toxic cholesterol oxides identified (cholestanetriol and 25-hydroxycholesterol) were then used for all further bioassays. In our study, the granulosa cells may have developed a tolerance to 7-ketocholesterol, which has previously been reported in hepatic cells (Erickson *et*

al., 1977). It has been reported that 7-ketocholesterol can be metabolised by cytochome P_{450} enzymes, to more polar and less toxic derivatives (Erickson *et al.*, 1977). Cytochome P_{450} enzymes have been detected in porcine granulosa cells (Benoit and Veldhius, 1996), which may account for the non-toxic effect seen in the present study. This may be important for two reasons. Firstly, 7-ketocholesterol was found to be the most abundant cholesterol oxide in milk powders in the present study, and by others (Nourooz-Zadeh and Appelqvist, 1988; Chan *et al.*, 1993). However, it's toxic effect *in vivo* (Erickson *et al.*, 1977) and on ovarian granulosa cells may be minimal. Secondly, it has been reported that humans can selectively absorb COPs, with 7-ketocholesterol being absorbed to the greatest extent (Emanuel *et al.*, 1991). Other studies, however, have shown inhibitory effects on cell viability of 7-ketocholesterol in cell types such as vascular smooth muscle and endothelial cells (Peng *et al.*, 1978), fibroblasts (Sevanian and Peterson, 1986), macrophages (Baranowski *et al.*, 1982) and CHO cells (Esterman *et al.*, 1983).

Mechanism of toxicity of COPs on porcine ovarian granulosa cells

Growth inhibition by cholestanetriol and 25-hydroxycholesterol was observed using trypan blue and MTT assays as viability indices (Fig. 5.6). The results indicate that when cells were exposed to either cholestanetriol or 25-hydroxycholesterol for 24 h, both plasma membrane and mitochondrial membrane integrity were significantly ($p < 0.001$) reduced (34 and 52 %, respectively). A number of mechanisms of COPs toxicity have been proposed (Smith and Johnson, 1989; Addis and Warner, 1992). It is possible that the reduced viability observed when granulosa cells were treated with COPs in the present study was the result of substitution of cholesterol in membranes by COPs. Uptake of 25-hydroxycholesterol has previously been reported, where 25-hydroxycholesterol replaced 75 % of the cholesterol in human fibroblasts *in vitro* (Peng and Taylor, 1984). Cholestanetriol has also been reported to displace free cholesterol from cell membrane in porcine kidney cells (Mahfouz *et al.*, 1995). The introduction of more hydrophilic groups into the biological membrane may alter physical configuration as well as function of the membrane (Peng and Taylor, 1984). Plasma membranes of cells exposed to COPs have been

reported to contain a significantly diminished cholesterol/phospholipid ratio (Peng and Morin, 1992). Such alterations can result in changes in the permeability of the cells to water and cations and eventually in a profound disturbance of membrane functions resulting in accumulation of intracellular calcium (Sevanian and Peterson, 1986), osmotic permeability and cell death (Streuli *et al.*, 1981). An inverse relationship has been observed between insertion of cholesterol oxides into membranes and osmotic fragility of the membranes (Streuli *et al.*, 1981). Proteins that have important cellular functions, such as transporters, receptors and enzymes (DePierre and Karnovsky, 1973) are embedded in the cholesterol-phospholipid bilayer of cell membranes. COPs which alter membrane fluidity may, therefore, affect positioning of membrane proteins and alter membrane-associated functions (Streuli *et al.*, 1981; Peng and Morin, 1992). One such protein which is affected is 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), an important rate- limiting enzyme in cholesterol biosynthesis (Kandutch and Chan, 1977; Erickson *et al,* 1977; Clare *et al.*, 1995). Consequently, cellular cholesterol may be decreased and the cholesterol available for membrane synthesis may be depleted. Granulosa cells, while having the capacity to synthesise cholesterol at certain stages in their development, also use LDL-derived cholesterol or theca cell-derived cholesterol as substrates for their steroid biosynthetic activities. For this reason, should inhibition of HMG-CoA reductase in granulosa cells occur as a result of cholesterol oxide administration, its effect would not give rise to the reported changes in cellular characteristics, such as altered mitochondrial integrity, calcium accumulation, cell death and osmotic fragility. COPs have also been reported to inhibit DNA and protein synthesis (Peng and Taylor, 1984), which may have been a cause of the reduction in cell numbers in cholesterol oxide-treated cells in this study.

Trypan blue assay and MTT assay correlation

A notable difference exists in the results between the effect due to COPs administration as determined by the trypan blue exclusion dye assay and the MTT assay. The MTT assay and the viable cell count assay were expected to correlate (Mosmann, 1983), but the MTT assay appeared to underestimate the growth

inhibitory effect of COPs compared to the viable cell count assay. The MTT assay (Mosmann, 1983) is based on the reduction of tetra-zolium salt (MTT) to a coloured formazan product by reducing enzymes present only in metabolically active cells. Succinate dehydrogenase is reported to be involved in the reduction (Jabbar *et al.*, 1989). The trypan blue assay, on the other hand, is based on uptake of dye by cells whose cell membranes are damaged, with the viable cells 'excluding' the dye. Several factors may have led to the difference in the results of the two assays. It may be that cell injury can actually induce mitochondrial activity. This has previously been reported (Jabbar *et al.*, 1989) where a 40 % increase in mitochondrial activity was observed after treatment of human lung cancer cell lines with interferon. In addition, no evidence exists that mitochondria are the only site of MTT reduction in the intact cell (Jabbar *et al.*, 1989).

Physiological concentrations of COPs

The absorption and delivery of COPs thoughout the body are important considerations. There are, however, limited studies on the dietary absorption of COPs. Steinberg *et al.* (1958) reported a greater than 70 % absorption of cholest-4-ene-3-one in rats, while Kikuchi *et al.* (1968) showed that the absorption of cholestanetriol was approximately 7 % and when cholesterol was administered simultaneously, it's absorption rate could be almost doubled. The mode of transport of COPs in plasma is also of interest, and they been reported to be localised in low-density lipoproteins (LDL) (56 %) and very-low-density-lipoproteins (VLDL) (34 %) while high-density-lipoproteins (HDL) contained only 10 % (Peng *et al.*, 1982) totalS COPs. The preferential transport of COPs in LDL and VLDL may be the basis for the more potent atherogenicity of these lipoprotein species (Carpenter *et al.*, 1993; Hughes *et al.*, 1994). Hughes *et al.* (1994) reported that the most toxic fraction of ox-LDL (oxidised low density lipoproteins) is 7-ketocholesterol. Peng *et al.* (1982) found that 24 h after ingestion, 25-hydroxycholesterol remained in the LDL and VLDL fractions, whereas Emanuel *et al.* (1991) found a residence time of approximately 3 h for the isomeric epoxides, 7β-hydroxycholesterol and 7-ketocholesterol in chylomicrons. The latter attributed the low residence time to the

relatively polar nature of COPs compared to sterol esters or triglycerides and the residence of COPs within the surface monolayer of plasma lipoproteins. Post-prandial plasma concentrations of total COPs (fed to 10 subjects at a concentration of 230 μg/g total COPs) were approximately 2-16 μg/ml after 3 h, after which they were cleared. The total plasma concentration of COPs was approximately 5 to 10-fold higher than that of chylomicrons, which suggested a rapid transfer of COPs from chylomicrons to other lipoproteins and/or plasma (Emanuel *et al.*,1991). It was apparent that plasma concentration varied among subjects fed the same quantity of COPs, suggesting individual differences in absorption (Emanuel *et al.*, 1991). Based on data obtained from Emanuel *et al.* (1991), consumption of approximately 30-40 μg/g COPs might be expected to yield a 1 μg/ml level in the blood. Hence, the level in the present study (2.5 μM) was deemed to be equivalent to 1 μg/ml in plasma.

Effects of COPs on exogenous (α-tocopherol) and endogenous antioxidants (SOD, CAT and Gpx)

A significant ($p < 0.05$) protective effect of α-tocopherol on cell viability was observed when administered simultaneously with both cholestanetriol and 25-hydroxycholesterol (Fig. 5.7). Previous reports have demonstrated a protective effect on cell viability when α-tocopherol was incubated simultaneously with COPs *in vitro* (Weitburg *et al.*, 1985; Wilson *et al.*, 1995). It has been reported that α-tocopherol functions as a protector against peroxidation *in vivo*. Being hydrophobic, it tends to accumulate within the membrane, so it can both quench and react with singlet oxygen, hydroxyl and superoxide radicals (Descampiaux *et al.*, 1996). However, its major antioxidant action in biological membranes is to react as a scavenger with lipid peroxy radicals, donating hydrogen and thereby terminating the chain-reaction of peroxidation (Halliwell and Gutteridge, 1989). Weitburg *et al.* (1985) reported that when CHO (chinese hamster ovary) cells were cultivated with α-tocopherol and then exposed to a cell-free oxidising system, little chomosomal damage occurred, thereby indicating that α-tocopherol is membrane associated to provide protection against oxidative stress. Typical plasma levels of α-tocopherol in humans have been reported to range from 6.6 - 15.0 (mean 9.6) μg/ml in plasma and

from 0.9-1.8 (mean 1.4) μg/ml in red blood cells (Chow, 1979; Lucesoli *et al.*, 1994). While the level of antioxidants added to the milk prior to spray-drying was not sufficient to abolish cholesterol oxidation as outlined in Chapter 4, the level of α-tocopherol added in the *in vitro* studies appeared to be sufficient to scavenge the free radicals produced as a result of cell injury due to the presence of COPs.

The level of lipid peroxidation in ovarian granulosa cells in the present study was assessed by the TBARS assay (Fig. 5.8). No significant increase in TBARS was observed after COPs administration with respect to the control. These results contrast with those of Wilson *et al.* (1995) who reported an increase in TBARS levels when newborn rat kidney (NRK) cells were incubated with COPs (5- 50 μM) for 72 h. Since the antioxidant defence enzymes (SOD and CAT) were increased on exposure to COPs in the present model system, it may be that these activities protected the granulosa cells from peroxidation damage. It may also be that the incubation period with COPs in the present study was not sufficiently long to result in significant lipid peroxidation.

In porcine ovarian granulosa cells, both SOD and CAT activities were increased significantly ($p < 0.01$) (Figs. 5.9-5.10) whereas a slight decrease was seen in GPx activity (Fig. 6.11) when COPs were administrated at a concentration of 2.5 μM. Wilson *et al.* (1995) found that SOD and CAT were also induced in chicken embryo fibroblast cells on exposure to 5- 50 μM COPs (7-ketocholesterol, 25-hydroxycholesterol and cholestanetriol). SOD action results in the accumulation of hydrogen peroxide, which in turn is removed by CAT and GPx. Since GPx actually decreased slightly, though not significantly, in porcine ovarian granulosa cells after exposure to COPs, it is possible that any hydrogen peroxide produced may have been eliminated from cells though the activity of CAT alone, and thus the COPs did not induce the activity of GPx in the granulosa cells. A decrease in GPx activity on incubation with toxic paraquat was observed by Lawlor and O' Brien (1994). They concluded that paraquat did not affect the thee antioxidant enzymes in a consistent manner: it induced SOD and CAT and inactivated GPx activity. This effect has also been seen in human hepatoma Hep 3B cells treated with lindane, an organochloride

insecticide, which is known to elicit free radical formation during it's metabolism in mammalian organisms (Descampiaux *et al.*, 1996).

Since antioxidant enzyme activities in granulosa cells were induced by COPs and restored to control levels in the presence of α-tocopherol, this model may be used in the future for screening food extracts for factors that enhance oxidative stress tolerance.

5.7 CONCLUSIONS

The effects of a number of COPs found in whole milk powder on porcine ovarian granulosa cells *in vitro* were investigated. 25-hydroxycholesterol, cholesterol-α-epoxide and cholestanetriol (2.5 μM) were found to be the most toxic, while similar concentrations of cholesterol and 7-ketocholesterol had no inhibitory effect on cell viability. 25-hydroxycholesterol significantly increased the activity of SOD ($p < 0.001$) and CAT ($p < 0.001$) while cholestanetriol induced a significant ($p < 0.01$) increase in SOD activity only. GPx activity decreased slightly, though not significantly, on exposure to 2.5 μM 25-hydroxycholesterol and cholestanetriol. The toxicities of 25-hydroxycholesterol and cholestanetriol were reduced by simultaneous incubation with 1 μM α-tocopherol. Finally, the lack of evidence implicating COPs-induced lipid peroxidation as the cause of cell death in this study is indicative of a sufficient antioxidant defence system in porcine ovarian granulosa cells.

CHAPTER 6

FINAL DISCUSSION AND CONCLUSIONS

Despite the current surge of research on COPs, the exact mechanisms of cholesterol oxidation remain unclear. However, in view of the wide range of processing operations employed within the food industry, it is obvious that the potential for cholesterol oxides to form in cholesterol-containing foods is very real, with some foods being more susceptible to cholesterol oxidation than others. Cholesterol oxidation has a number of undesirable biological effects and hence it is of concern to food scientists and consumers. Research on the occurrence of cholesterol oxides is in its infancy, although there have been vast improvements in the methodologies used in their detection over the past few years. The daily food intake for an average person is about 500 g (Van de Bovenkamp *et al.*, 1988) with a daily cholesterol intake averaging 280 mg/day (Sarantinos *et al.*, 1993). Thus, the typical level of oxysterols in the average diet is at most a few mg per day (Van de Bovenkamp *et al.*, 1988). Whether such amounts contribute to the development of chonic diseases is at present unknown. The aims of the current study were 4-fold: (i) to establish a valid and accurate analytical method for detection of COPs in whole milk powders, (ii) to investigate the effect of animal feeding regimen on levels of COPs in whole milk powders (iii) to determine the effect of particular processing conditions on lipid and cholesterol oxidation in whole milk powder and (iv) to ascertain the *in vitro* effects of COPs on cell growth and oxidative status.

The quantification of cholesterol oxides from biological materials and foods is difficult because their isolation is frequently impeded by large amounts of interfering cholesterol, triglycerides, phospholipids and other lipids. Whole milk powders contain approximately 0.1 % (w/v) cholesterol (Chan *et al.*, 1993). Cholesterol oxidation in whole milk powders has not been studied to the same extent as in egg products (Morgan and Armstrong, 1992) and a wide variety of methodologies have been used leading to large discrepancies in the results reported. COPs levels differing by over a 1000-fold, from 250 ng/g (Rose-Sallin *et al.*, 1995) to 300 μg/g (Chan *et al.*, 1993) have been reported in stored whole milk powder. Such variation presently gives rise to controversy, and the precise quantification of COPs remains a challenging task, especially in foods containing low levels of these compounds. In the present study, GC was used as the analytical method for COPs detection and

measurement. The setting up of a valid method entailed determining the recovery, precision, linearity and reproducibility of the method. Solid phase extraction was used prior to sample injection as a sample clean-up procedure, which resulted in 'cleaner' chomatograms. MS was used for confirmation, since FID alone cannot distinguish between co-eluting peaks which can present problems in cholesterol oxide detection. The survey of commercial milk powders showed that the level and type of COPs found in whole milk powder during storage may be greatly influenced by the fat content of milk and the extent of pre-heating of milk prior to spray-drying. In order to establish a valid method worldwide, the method should be validated via participation in a round robin test. This would establish the accuracy, recovery and reproducibility of the method and permit any methodology errors to be minimised so that food scientists and toxicologists could accurately establish dietary COPs.

Having established a method for reliably quantifying cholesterol oxides in whole milk powders, we subsequently investigated the effects of animal feed quality on the extent of cholesterol and lipid oxidation during processing and storage of whole milk powder.

Milkfat composition may be rapidly altered by nutritional manipulation and may therefore be a more appropriate means of improving milk and dairy product quality (Sutton, 1989) than genetic means. Therefore, modification of animal feeding regimen was used in the second stage of our study as a means to investigate the role of nutrition in offering protection against oxidation of whole milk powder. Feed provides the nutrients that are the precursors, either directly or indirectly, of the principal milk solids and particular diets may result in the production of greater volumes of milk (Sutton, 1989). In our study, the grass plus concentrate-fed herd (S) received a standard concentrate-fed at 3 kg/cow/day, which is the standard dosage (Murphy, 1996 Personal Communication). The concentrate fed to the herd S included protein, carbohydrate, lipid, crude fibre and minerals and vitamins. The grass-fed herd (R) was fed the Moorepark standard grass supply without a concentrate. It has previously been reported that of the principal solid constituents of milk, the concentration of fat can be altered by approximately 3 % by nutritional

means while protein can be altered by only approximately 0.6 % and lactose scarcely at all (Sutton, 1989). In the present study, however, no significant differences were observed in the fat levels or milkfat composition, while a significant ($p < 0.05$) increase was observed in the total protein levels, casein and whey levels in milk of the grass plus concentrate-fed herd compared to the grass only-fed herd. The levels of lipid and cholesterol oxidation in the resultant whole milk powders were lower in higher quality concentrate-fed diet, indicating that animal feeding regimen does have a protective effect on lipid and cholesterol oxidation in whole milk powder. The improved level of protection against oxidation in milk powder manufacture from milk of the grass plus concentrate-fed herd was attributed to a number of factors. Since both whey and casein have reported antioxidant activity (Taylor and Richardson, 1980; Allen and Wrieden, 1982), and were elevated in the milk of the grass plus concentrate-fed herd, the protective effect observed may have been due to the total antioxidant activity of the casein and whey fractions of milk. Higher levels of free sulphydryl groups were observed in whole milk powder of the grass plus concentrate-fed herd which may have, in part at least, contributed to the reduced levels of lipid and cholesterol oxidation observed. α-Tocopherol was included in the concentrate at a standard level of 22.5 IU/cow/day which may also have contributed to the protective effect in the grass plus concentrate-fed herd milk. A shortcoming of this study was the absence of whole milk powders produced from a control herd fed the Moorepark standard grass supply. The grass only-fed herd differed from the standard Moorepark standard grass management system in having a lower land allocation, a lower stocking rate and no concentrate supplementation. Therefore, any differences observed between the two herds used in this study, i.e. grass-fed and grass plus concentrate-fed could not be related to a control herd. Future work in this area might investigate the effects of increasing the levels of α-tocopherol in the feed and/or intraperitoneal injection of vitamin E, and assessing the relationship between the resultant vitamin E levels in the milk and the oxidative stability of resultant whole milk powder.

It was previously reported that formation of COPs in dried foods of animal origin can be minimised by developing improved methods of processing, packing and

storage (Chan *et al.*, 1993). The third section of this study investigated the effects of pre-heat temperatures, packing conditions and storage temperatures on lipid and cholesterol oxidation in whole milk powder. A number of physicochemical and functional properties of whole milk powder were investigated in fresh and stored powders. High pre-heat treatment caused significantly ($p < 0.001$) greater protein denaturation than low pre-heat treatment. Organoleptic properties of the powders were also measured and high-heat whole milk powder, vacuum-packed and stored at 15° C gave the best flavour score (90). Overall, low-heat whole milk powder resulted in reduced oxidative stability after 12 months storage as indicated by increased PV, TBARS and COPs in these powders. Vacuum-packing markedly reduced lipid and cholesterol oxidation compared to sachet-packing while storage at 15° C resulted in reduced lipid and cholesterol oxidation compared to storage at 30° C. However, oxidation was not completely eliminated in vacuum-packed powders. Further work on the effect of flushing with an inert gas such as nitrogen prior to packing is warranted to investigate its role in further protection against lipid and cholesterol oxidation.

α-Tocopherol (added at 500 μg/g lipid and 1000 μg/g lipid) inhibited lipid oxidation at ambient temperatures (15° C), but did not significantly reduce cholesterol oxidation under any conditions investigated, suggesting that a theshold limit of cholesterol oxides for protection by α-tocopherol may exist. α-Tocopherol was added both to milk prior to spray-drying and to powder following manufacture but only the former mode of addition was found to be effective at reducing lipid oxidation, effective antioxidant activity presumably arising from its incorporation into the milk fat globule membrane. Other studies have shown that antioxidant addition reduced lipid oxidation in whole milk powder (Tuohy, 1987; Chan, 1992). Further research is needed to investigate the effects of varying levels of α-tocopherol, and indeed other natural antioxidants, on cholesterol and lipid retardation. Since addition of antioxidants to milk during processing is not permitted in Ireland (O' Shea, 1996 Personal Communication), further research is needed to investigate the potential for increasing levels of of antioxidants in milk by dietary means. While a lot of data exists on the levels of COPs in food products such as

spray-dried egg products (Tsai and Hudson, 1984), meat products (Higley *et al.*, 1986; Monahan *et al.*, 1992; Buckley *et al.*, 1995) and dairy products (Chan *et al.*, 1993; Rose-Sallin *et al.*, 1995), there is still a need to investigate other cholesterol-containing foodstuffs, such as infant formulae, confectionary and convenience foods. It would be of immense benefit to establish a data base on levels of cholesterol oxidation products in Irish foodstuffs, as has been done for foodstuffs in Sweden (Nourooz-Zadeh and Appelqvist, 1988) and Australia (Sarantinos *et al.*, 1993).

Lipid and cholesterol oxidation were positively correlated during storage of whole milk powder. This has also been previously reported in pork (Monahan *et al.*, 1992), whole milk powder (Chan *et al.*, 1993) and dairy spreads (Nielsen *et al.*, 1996). However, the nature of the correlation in pork and whole milk powder was quite different to that observed in dairy spreads. In the former, cholesterol oxidation mirrored development of secondary oxidation while in the latter cholesterol oxidation reflected primary lipid oxidation. In view of the relatively non-specific nature of the TBA assay, it may be concluded that the COPs assay might be as appropriate, if not more appropriate than a measure of TBARS for monitoring lipid oxidation, in particular foodstuffs such as pork and milk powders.

Several cholesterol oxidation products have been implicated in a number of adverse biological effects including atherogenesis, cytotoxicity and carcinogenesis (Kumar and Singhal, 1991). There is, however, a relative dearth of information on their effects on major steroidogenic tissues other than liver. Gonadal tissues are actively steroidogenic using cholesterol as precursor. Granulosa cells in the ovaries are responsible for the production of C18 oestrogens. The 19-hydroxylase is, like other hydroxylases found in mitochondria and endoplasmic reticulum of steroid-producing tissues, a mixed function oxidase having cytochome P_{450} at the active site. In view of the active cytochome P_{450} enzyme system in granulosa cells, and its likely dependence on the oxidative status of cells, it was of interest to investigate whether viability of ovarian granulosa cells might be adversely influenced by cholesterol oxides via damage to cellular and subcellular endoplasmic reticular membranes and/or via altered function of key protein enzymes involved in antioxidative defence. Porcine ovarian granulosa cells were used as a model system to assess the biological

effects of the following cholesterol oxidation products: 7-ketocholesterol, α-epoxide, 25-hydroxycholesterol, cholestanetriol. The *in vitro* effects of purified cholesterol were also investigated. 25-hydroxycholesterol, α-epoxide and cholestanetriol were found to be the most toxic, while cholesterol and 7-ketocholesterol had no inhibitory effect on cell growth. 25- hydroxycholesterol induced significant increases in SOD activity ($p < 0.001$) and CAT activity ($p < 0.001$) while cholestanetriol induced a significant ($p < 0.01$) increase in SOD activity only. GPx activity was reduced by exposure to COPs. The data reported in Chapter 5 suggest that cholesterol oxides generated superoxide radicals and hydrogen peroxide in granulosa cells but that the cells were, in part able to adapt by inducing antioxidant defence enzymes. That cell death occurred, however, suggests some deficiency in the levels of antioxidant defences. There was no significant effect on lipid peroxidation as indicated by TBARS levels. Since antioxidant enzyme activities were induced by COPs in the present model system, this model system could be used to compare the relative defensive effects of an exogenous antioxidant, α-tocopherol. The toxicity of 25-hydroxycholesterol and cholestanetriol was reduced by incubation with α-tocopherol (1 μM). Relatively low concentrations of COPs were used in these cell culture studies. However, based on extrapolations from the only human feeding study carried out by Emanuel *et al.* (1991), this concentration of COPs in cell culture (1 μg/ml) would be expected to simulate the plasma COPs levels obtained after ingestion of 1 g low-heat-treated milk powder sachet-packed and stored at 30° C. Further work in this area could focus on the effect of COPs at higher concentrations, to determine the LD_{50} for each of the COPs found in whole milk powders. The exact mechanism of cell toxicity by COPs administration still remains unclear. It may be that cholesterol is displaced from the cell membrane by COPs, thus altering cellular integrity and function. It would be of interest to investigate levels of HMG-CoA reductase in the porcine ovarian granulosa cells to determine rate of cholesterol biosynthesis and whether inhibition of mevalonate and cholesterol synthesis may have led to cell death via interference with progression though G1 (membranogenesis) and S phases (DNA replication) of the cell cycle. Furthermore, analysis of the oxysterol profile of cells exposed to cholesterol oxides would have

yielded information on the capacity of these cells not only to take up oxysterols but also to generate oxysterols by secondary oxidation.

Further biochemical research is needed to elucidate the exact mechanism of cholesterol toxicity. With regard to the current study where cell number and mitchondrial integrity were used as indicators of cell viability, further work could focus on LDH release to verify results from trypan blue and MTT assays. In addition, indices of cell function including generation of ATP, measurement of redox potential, extent of ^{3}H thymidine incorporated into DNA and extent of ^{14}C leucine incorporation into protein could be investigated. Very few studies have been performed to investigate the effect of COPs *in vivo* and further research is therefore required to ascertain the levels of COPs *in vivo* as a result of ingestion of dietary COPs. COPs have been detected in atheroma samples but not in normal aorta (Carpenter *et al.*, 1993) and hence the possibility of phenotyping serum lipids for COPs may be a useful diagnostic marker. The levels of COPs administered to cells in the present study (1 μg/ml) were lower than has been reported in human plasma (2-16 μg/ml) (Emanuel *et al.*, 1991) and no significant lipid peroxidation was observed (as indicated by TBARS) in cultured porcine ovarian granulosa cells. This would imply that the levels of COPs found in whole milk powders in this study may not cause a significantly toxic effect *in vivo*.

CHAPTER 7

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APPENDICES

APPENDIX I

Questionnaire on Whole Milk Powder Survey

Cholesterol Oxidation Products (COPS) Questionnaire on WMP

Company name:

Contact person:

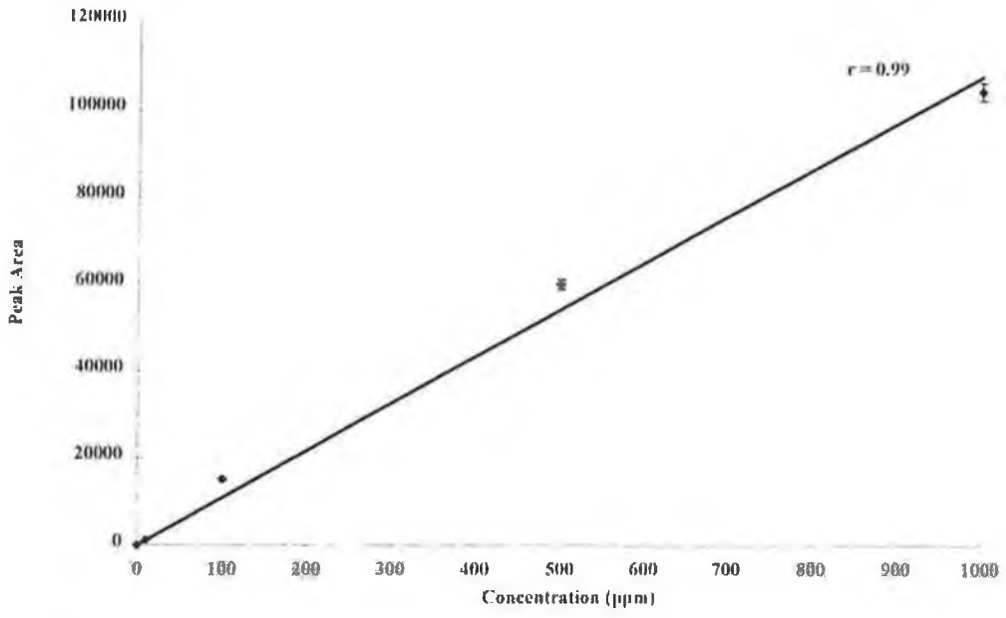
Please tick appropriate box and include necessary data:

Heat Stable Class	Production Date	Storage Temp.	Atomizer Type (Disc/Nozzle)
Low			
Med.			
High			

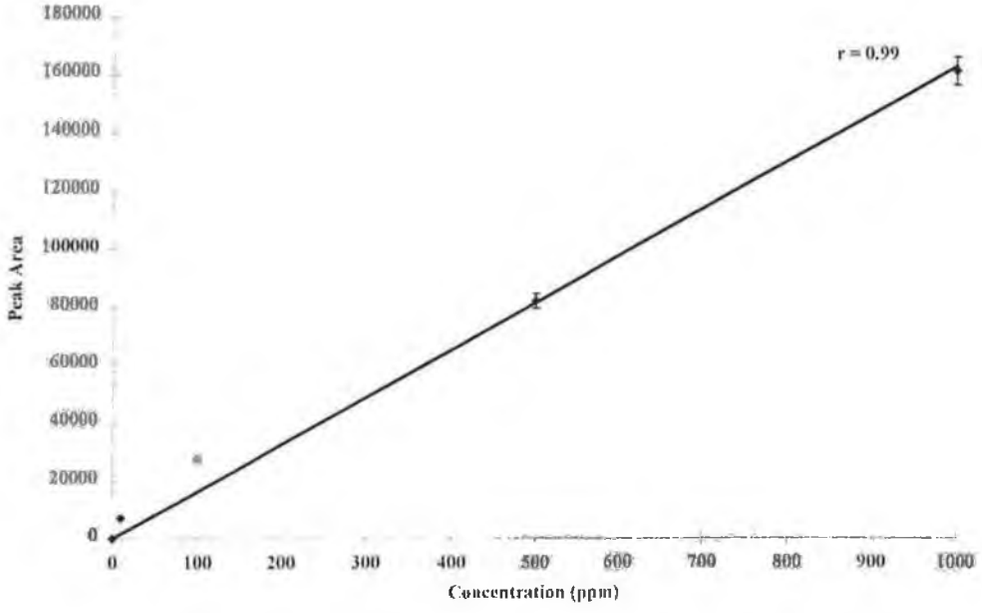
APPENDIX II

Standard Curves of Cholesterol Oxidation Products (GC-MS)

(a)

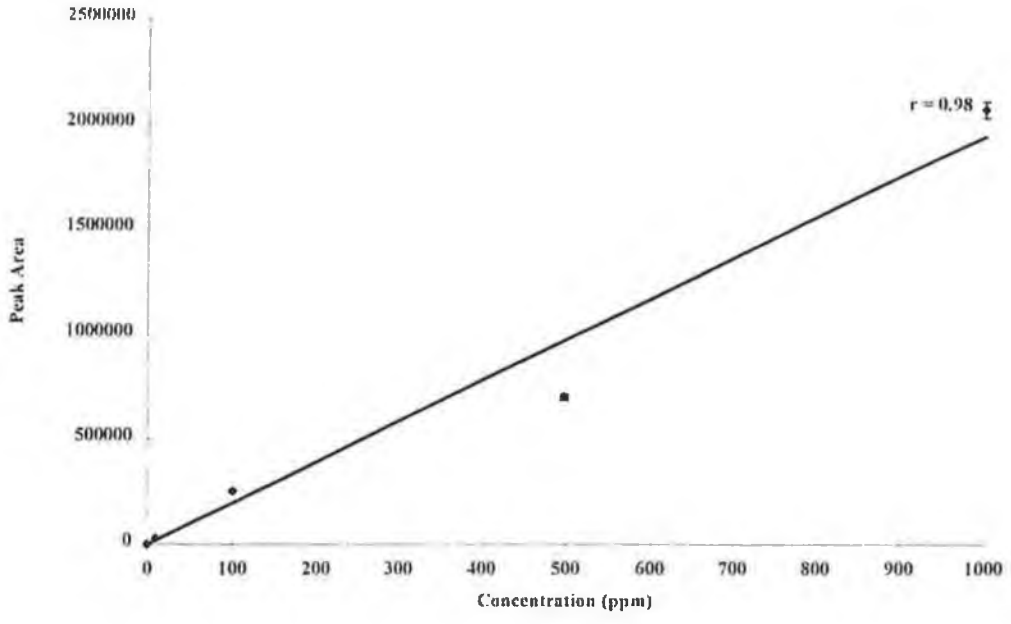


(b)

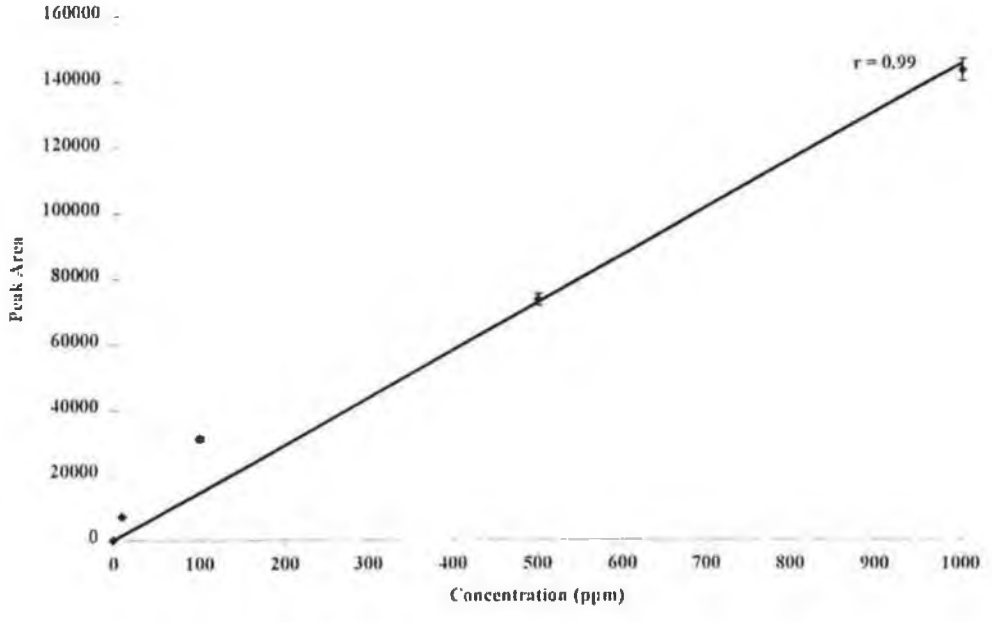


Standard curves of (a) α-epoxycholesterol and (b) 25-hydroxycholesterol.

(a)



(b)



Standard curves of (a) cholesterol and (b) 7-ketocholesterol.

APPENDIX III

Procedure for Flavour Scoring in Whole Milk Powder

THE BORDEN COMPANY LIMITED

PROCEDURE TITLE: PROCEDURE FOR FLAVOUR SCORING

PROCEDURE NO : 1.8 **REVISION NO :** RO0 **PAGE 1 of 2**

APPROVED BY	TITLE	AUTHORISED BY	TITLE
_____	_____	_____	_____
_____	_____		
_____	_____		
_____	_____	**DATE:**	_____

1.0 PURPOSE

1.1 To determine and assign a flavour score for milk powder.

2.0 Reagent

2.1 Demineralized water.

3.0 Equipment and Ancillaries

3.1 Analytical Scales.

3.2 Thermometer.

3.3 Glass beakers (200 ml).

3.4 Mixing Equipment: Instant Powder - Fork.
Regular - Blender.

4.0 Procedure

4.1 Responsibility: Laboratory Assistant.

4.2 Take a min.of three samples per batch for Incoming Inspection (first, middle and last bin).
Take one can, twice per shift (a.m. and p.m.), for Final Inspection.
Take samples for storage as stated in Procedure No. 1.20.

4.3 Pour 90 ±0. 5 ml of 25 ± 2^{o}C. demineralized water into the beaker.

4.4 Weigh 13 ± 0.5g of powder on analytical scales.

4.5 Put instant powder into the beaker and mix with a fork for 10 seconds.
Put regular powder into the blender and mix for 90 seconds.

4.6 Allow reconstituted sample to stand for 1 hour at room temperature before flavouring.

4.7 Prepare a sample tray for each panelist by placing a 50ml beaker into each well

4.8 Sub-divide each reconstituted sample, according to the number of panelists participating, into respective 50ml beaker in the sample tray, i.e. sample 4 would be sub-diivided into number 4 well in each of the sample trays.

4.9 Record date, time, code, powder type and sample number in Flavour Log Books before flavour score sampling begins.These must be recorded for the Storage samples as well.

4.10 Panelists will flavour the samples and assign them a score on their Flavour Score Sheet (BML/S049), without consultation with any of the panelists.

4.11 Transfer results from Incoming > Laboratory Report Sheet
Final > Green Book
Storage > Storage Book

5.0 ASSIGNING SCORE

5.1 The flavour and odour should resemble closely that of freshly made milk sample and any variations should be indicated on Flavour Score Sheet (BNML/S049).

5.2 The value of the test lies in the ability of the panelist to recognise the normal flavour and odour characteristics of a good quality powder and to appreciate any variations that may occur.

5.3 The flavour score is reported by a numbering system as follows:

5.3.1 Perfect, slightly cooked flavour.	90
5.3.2 Good: no off flavour, not quite as fresh tasting as 90.	85
5.3.3 Fair; no strong off flavour, but less fresh tasting than 85.	80
5.3.4 Objectionable; some off flavour.	75

5.4 An experienced, discerning taster will be capable of assigning some score values which lie within the above figures (88, 83, etc.).

5.5 Off flavours may be identified as:

5.5.1 Acid
5.5.2 Bitter
5.5.3 Chalky
5.5.4 Burnt
5.55 Feed
5.5.6 Flat
5.5.7 Metallic
5.5.8 Oxidized or tallowy
5.5.9 Rancid
5.5.10 Salty
5.2.11 Stale
5.2.12 Weedy

6.0 OFF SPECIFICATION ACTION

6.1 Refer to Supervisor.

APPENDIX IV

Statistical Analysis of the Effects of Animal Feed Quality, Pre-Heat Temperature, Packaging Conditions, Storage Temperature and Period of Manufacture on Lipid and Cholesterol Oxidation Products in Whole Milk Powder

Effect of animal feed quality, pre-heat, packing and storage temperature on TBARS levels after (a) 2 months, (b) 4 months, (c) 6 months storage and (d) 12 months storage.

(a)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	0.001	N.S.	N.S.	N.S.
Pre-heat	0.001	0.01	0.01	0.01
Packing	0.05	0.05	N.S.	0.05
StorageTemperature	0.001	0.001	0.05	0.01

(b)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	0.05	0.05	N.S.	N.S.
Pre-heat	0.001	0.001	0.001	0.001
Packing	0.001	N.S.	N.S.	N.S.
StorageTemperature	0.001	N.S.	N.S.	N.S.

(c)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	N.S.	0.01	N.S.	N.S.
Pre-heat	0.001	0.001	0.01	0.01
Packing	0.001	0.001	0.01	0.01
StorageTemperature	0.001	0.001	0.05	0.01

(d)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	0.001	0.01	N.S.	0.001
Pre-heat	0.001	0.001	0.05	0.01
Packing	0.001	0.001	0.001	0.01
Storage Temperature	0.001	0.001	0.001	0.001

Effect of animal feed quality, pre-heat, packing and storage temperature on COPs after 12 months storage.

Effect	Period 1	Period 2	Period 3	Overall
Animal feed quality	0.01	0.01	0.01	0.01
Pre-heat	0.001	0.001	0.001	0.001
Packing	0.001	0.001	0.001	0.001
Storage Temperature	0.001	0.001	0.001	0.001

APPENDIX V

Publications

Effect of animal feed quality and pre-heat treatment on PV and TBARS in fresh whole milk powder.

Effect	Oxidation index	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	PV	0.05	0.05	N.S.	N.S.
Pre-heat		0.001	N.S	N.S.	N.S.
Animal Feed Quality	TBARS	N.S.	0.05	N.S.	N.S.
Pre-heat		N.S.	N.S.	N.S.	N.S.

Effect of animal feed quality, pre-heat, packing and storage temperature on PV levels after (a) 2 months, (b) 4 months, (c) 6 months storage and (d) 12 months storage.

(a)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	N.S.	N.S.	N.S.	N.S.
Pre-heat	0.01	0.01	N.S.	0.01
Packing	0.001	0.001	0.05	0.01
StorageTemperature	0.01	0.01	0.01	0.01

(b)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	N.S.	N.S.	N.S.	N.S.
Pre-heat	0.001	0.001	0.05	0.01
Packing	0.05	0.001	N.S.	0.05
StorageTemperature	0.001	0.001	N.S.	0.01

(c)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	0.001	N.S.	N.S.	N.S.
Pre-heat	0.01	0.01	0.05	0.01
Packing	0.001	0.01	0.001	0.01
StorageTemperature	0.001	0.001	0.001	0.001

(d)

Effect	Period 1	Period 2	Period 3	Overall
Animal Feed Quality	N.S.	0.01	N.S.	N.S.
Pre-heat	0.01	0.001	0.05	0.05
Packing	0.001	N.S.	N.S.	N.S.
StorageTemperature	0.001	0.05	0.05	0.05

PUBLICATIONS

- Mc Cluskey, S. and Devery, R. (1993). Validation of Chromatographic Methods for Detection of Cholesterol Oxides in Dried Foods. Trends in Food Sci. & Technology (4) pp 175-178.

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- Mc Cluskey, S., Kelly, J., O'Brien, B., Stanton, C. and Connolly, J.F. (1995). Development of Cholesterol Oxidation Products in Whole Milk Powder. Proc. IDF Nutrition Week, Paris, 14-16 June.

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