CHAPTER II

THEORY

2.1 Milk Composition *(26)*

Milk is a complex fluid containing many components in several state of dispersion. Understanding its properties and the many changes that can occur requires through knowledge of all of the components and their effects on one another. The composition of individual components will be described in turn and comment made on the major aspects of their chemistry as they affect processing.

The major determinants are significant differences in the composition of milk from individual cows are cow breed, diet, age, and stage of lactation. For bulk milk, the major influence on composition is diet. A rough classification of the principal milk constituents with approximate percentages of contents is given in Table 2.1

Table 2.1 Approximate Composition of Milk.

Component	Average Content Percentage (w/w)
Water	87.3
Solid-no-fat	8.8
Fat in dry matter	31.0
Lactose	4.6
Fat	3.9
Protein	3.25
Casein	2.6
Mineral substances	0.65
Organic acid	0.18
Miscellaneous	0.14

2.1.1 Milk Fat

Milk fat is excreted in the form of small droplets which, in cow's milk, range in size from 1 to 12 μm in diameter with a mean size of about 3 μm. The mean size appears to be related to the fat content of the milk and the higher the fat content, the bigger the average size of the individual fat globule. During the excretion of the fat droplets from the secretory cells of the mammary gland, the globules are encapsulated in membrane material derived from the apical membrane. The milk fat globule membrane comprises largely of proteins and phospholipids and provides stabilization for the hydrophobic fat particles in the aqueous environment of the milk serum. The milk fat globule membrane is however a delicate structure and can easily be ruptured by either physical or thermal shock. When this happens accidentally or deliberately during the process for the manufacture of butter the fat globule churn and coalesce into a solid mass of fat. Less dramatic damage to the membrane may retain the physical stability of the globule but can render the fat susceptible to the action of lipoprotein lipase, a lipolytic enzyme present in great excess in raw milk. Lipolytic action leads to the hydrolysis of milk fat and the liberation of free fatty acid, resulting in the development of unacceptable rancid flavor and odor. For these reasons, raw milk must be carefully handled during all stage of transport and storage.

The density of the milk fat globule is much lower than that of the milk serum and, as a result, milk fat globule rise to form a fat rich phase, a process commonly called creaming. This density difference is used to separate milk fat from whole milk in high speed, continuous flow, and centrifugal cream separators.

Triacylglycerols are the predominant lipid class in bovine milk, accounting for 97-98 % of the total lipid. The remaining lipid classes are di- and mono- acylglycerols, phospholipids, free fatty acid, and its esters. The triacylgycerols in milk are in themselves exceedingly complex for, not only are many different types of fatty acid esterified to the glycerol, but the position at which fatty acid are attached may also vary.

Fatty acids in milk arise from three main sources; from the feed, from mobilization of reserve tissue and from *de novo* synthesis within the cow. The main source of lipid is from the feedstuff. The dietary lipid is ingested, and then hydrolyzed to free fatty acids in the rumen of the cow. In the rumen, unsaturated fatty acids are usually hydrogenated. The fatty acids then either enter the bloodstream to be deposited as reserve fat or to be metabolized for production of energy, or enter the mammary gland. Reserve fat may also be mobilized when the diet of the cow is energy deficient and, at such times, fatty acids from this source are incorporated in milk fat. A significant level of *de novo* synthesis of fatty acids also occurs in the mammary gland.

The composition of milk fat depends on the route from which it is derived. *De novo* synthesis results in the production of short chain fatty acids with four to fourteen carbon atoms (C_4-C_{14}) and in the production of some palmitic acid (C16), although palmitic acid is also derived from the blood supply. When the feedstuff is depleting of pre-formed fatty acid, *de novo* synthesis is enhanced and vice versa.

Milk fat would be saturated in nature since the reduction process in rumen converts unsaturated substrate to the saturated form. In fact, over 30 % of milk fat is unsaturated and the unsaturated fat is predominated by oleic acid (C18:1). The C18 acid enter the mammary gland mostly in the saturated form but within the gland there is specific C18:0 desaturase which converts stearic acid (C18:0) to oleic acid (C18:1). As a result, there is a significant amount of unsaturated lipid in milk fat. Table 2.2 showed fatty acids in cow milk.

As might be expected, the characteristics of milk fat are highly dependent on the nature of the pre-formed fatty acids available in the feedstuff. Thus it is feasible to manipulate the composition of milk fat by dietary means and, because the melting properties of the triglyceride are related to fatty acid composition, the physical properties of the butterfat can be controlled. For example, butter which can be spread directly from the refrigerator can be produced. Alternatively, butter which is physically stable at high temperature can be made. To date, commercial factors have precluded exploitation of such technology.

Table 2.2 Fatty acid composition of milk.

Fatty acid	Number of carbon: Number of double bond	% w/w of fatty acid/fat
Butyric acid	C4:0	3.7
Caproic acid	C6:0	2.0
Caprylic acid	C8:0	1.6
Capric acid	C10:0	2.6
Lauric acid	C12:0	3.3
Myristic acid	C14:0	8.7
Palmitic acid	C16:0	27.0
Stearic acid	C18:0	10.0
Oleic acid	C18:1	35.0
Linoleic acid	C18:2	4.5
Linolenic acid	C18:3	0.6

The chemistry of milk fat is dominated by two types of reaction, hydrolysis and oxidation. Hydrolysis, i.e. the liberation of free fatty acids, requires the presence of a lipase. As mentioned earlier, natural milk lipoprotein lipase is present in milk great excess but fortunately globules with an intact milk fat globule membrane are not susceptible to attack and the enzyme is readily inactivated by pasteurization. Spoilage bacteria provide another source of lipase and, in this case, the enzyme may by very heat stable. However, the levels of contaminants in the milk must exceed normal standards for such spoilage to occur and routine bacteriological control should prevent such an occurrence.

Oxidation of milk fat is not a serious problem but may limit the shelf-life of whole milk powders. Oxidation is catalyzed by light, and by contamination of the milk by trace levels of some metals such as copper.

Milk fat can be an important source of dietary energy. However, in recent years the role of dietary fat type on the incidence of arterial disease in adults has become the source of great controversy in nutritional and medical circles. Much of the

evidence relating diet to disease is epidemiological and it is difficult to draw firm conclusions. The most balanced view put by experts in the field acknowledges that over consumption of saturated fat may predispose some individuals to risk but that the consumption of modest levels of milk fat within a balanced diet is unlikely to have a deleterious effect.

2.1.2 Milk Protein

The proteins in milk fall into two distinct types, whey proteins and caseins. Whilst the relative proportion of whey protein to casein may vary over the season, typical mid-season values are shown in table 2.3. In the first few days of lactation and in late lactation, the whey protein content of milk may increase substantially and, at these times, proportion of blood serum proteins in the milk is elevated.

The caseins constitute over 80 % of the total protein and from a distinct family which may itself be subdivide into five main classes, the $alpha_{s1}$, $alpha_{s2}$, beta, gamma and kappa caseins.

Table 2.3 Protein distribution in skimmed milk.

Protein type	%
Casein	82.2
Whey protein	
Beta lactoglobulin	9.6
Alpha lactoglobulin	3.8
Bovine serum albumin	1.4
Minor components	3.0

The $alpha_s$ and beta casein are phosphoproteins with between 5 and 13 phosphoserine groups. These phosphoserine residues are the key to the unique properties of casein.

Gamma caseins are the result of post-secretory hydrolysis of beta casein. In good quality milk from cows in mid-lactation, gamma casein should constitute a very low proportion of the total casein fraction in milk certainly less than 5%. When gamma casein levels exceed this figure, proteolytic enzymes have been active in the milk, and the milk may be difficult to process into certain types of product. If the proteinase responsible for degradation is native milk proteinase, little prompt remedial action can be taken. However, the major source of proteolytic activity in milk arises from bacteriological contamination and, in this case; improvements in hygiene can be initiated to resolve the problem.

Kappa casein contrasts with the other caseins as it has one serine phosphate ester group and contains a charged carbohydrate moiety. This casein is insensitive to the addition of calcium.

In contrast to the caseins, the whey proteins are typical globular proteins which may be denatured on heating at temperatures above 65 °C. The major components are beta lactogloburin and alpha lactogloburin. Bovine serum albumin and immunoglobulins are also present, though at low levels in the milk from healthy cows in mid-lactation. In late lactation, or when mastitis is present in the mammary gland, 'leakage' of these blood-derived proteins may occur, leading to elevated levels in the milk.

The major whey contains sulphur amino acid which, on heating, may participate in sulphydryl exchange reaction leading to inter- and intra-molecular cross-linking.

The most important reactions of the milk proteins are those which involve destabilization of the casein micelles. The simplest reactions are those which occur as the pH of milk is reduced. At pH 4.6, the isoelectric point of the caseins, precipitation of the caseins (and any heat denatured whey protein) occurs. Although reaction may be reversed by increasing the pH of the milk to its starting value (around pH 6.7), reversal occurs slowly and the native form of the micelles is not recovered. Acidification is the basis of formation of many fermented products such as yogurt.

Milk protein has a very high nutritional value. The essential amino acid profiles of both casein and whey protein are shown in Table 2.4. When compared to the United Nations Food and Agricultural Organization (FAO) provisional scoring pattern for quality, both milk protein fractions score very highly. Casein is slightly poorer than whey protein in its content of sulphur amino acids (methionine and cysteine) but the complementary nature of the profiles of the two milk protein fractions is such that the nutritional value of whole milk is such that it is complementary to grain and soybean protein. For this reason, milk protein finds a ready use for fortification of the nutritional value of foods such as pasta.

Table 2.4 Essential amino acid profiles of milk proteins.

Amino acid	mg amino acid per gram protein			
	Casein	Whey protein	Skim milk	FAO provisional scoring pattern
Isoleucine	54	76	58	40
Leucine	95	118	99	70
Lysine	81	113	87	55
Methionine+Cysteine	32	52	36	35
Phenylalanine+Tyrosine	111	70	103	60
Threonine	47	84	54	40
Tryptophan	16	24	18	10
Valine	75	72	74	50
Total essential amino acids	511	609	529	360

2.1.3 Lactose

As shown in Table 2.1. Lactose is by weight the most abundant of the milk solids. Its concentration in milk is related to yield and may range from 4.2 to 5.0%. Low lactose contents are usually associated with milk from animals in very late lactation, or in those suffering from udder disease.

Lactose is a disaccharide comprised of alpha-D-glucose linked to beta-D-galactose as shown in Figure 2.1. The sugar may exist in two different crystalline forms, alpha and beta, which differ in their properties. The alpha form of lactose is less soluble (7% w/w in water at 15°C) than the beta form, though in solution an equilibrium mixture of both forms is established by a process known as mutarotation. This equilibrium mixture has solubility in water of about 17% at 15°C.

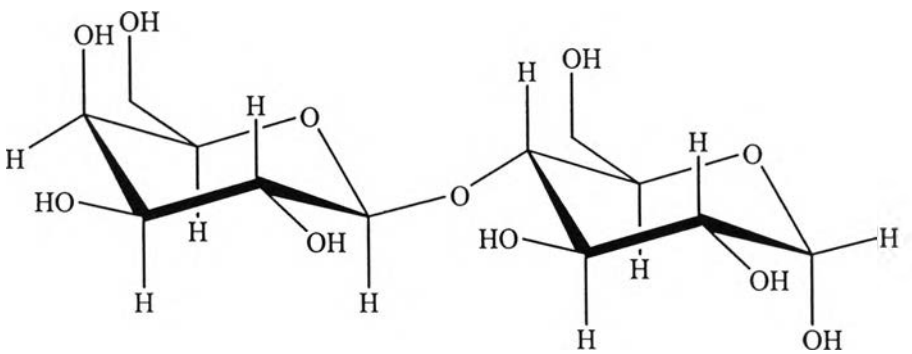

Figure 2.1 The structure of lactose.

Lactose is a reducing sugar and, in some circumstances, reacts freely with free amino groups in protein. The classical Maillard reaction is of this type. The Maillard reaction is characterized by browning but, in the earlier stages of the reaction, there is a significant loss of nutritive value as the essential amino acid; lysine reacts with reducing sugar. Furthermore, Maillard reaction results in flavor defects. The extent of the loss of available lysine depends on the severity of heat treatment, the pH of the milk product, and amount (and type, in the case of products where sugars are added) of reducing sugar.

Hydrolysis of lactose can lead to change in the properties of milk. Sweetness is enhanced and lactose crystallization no longer occurs in concentrates, an advantage for the production of frozen products. However, every molecule of lactose hydrolyzed yields two molecules of reducing sugar which are active in browning reactions. As a result, particular care must be taken to preserve the nutritive value of the modified milks.

2.1.4. Minerals in milk

As described in Section 2.1.2., the integrity of the casein micelles in milk is largely due to a network of calcium and phosphate which binds the alpha$_s$ together. In addition to calcium and phosphate (both serine bound and free), milk is rich in some monovalent ions and citrate. Typical values of the mineral content of milk are shown in Table 2.5. The minerals are partitioned between a colloidal and a diffusible or 'soluble' phase. The monovalent anions and cations are found almost exclusively in the soluble phases. About one-third of the total calcium and half of the total inorganic phosphate are unbound.

Table 2.5 Minerals in milk.

Mineral	Total (mmol/L)
Calcium	30.1
Magnesium	5.1
Sodium	25.5
Potassium	36.8
Chloride	30.3
Inorganic phosphate	20.9
Citrate	9.8

Seasonal changes in the total mineral content of milk are modest and are largely related to protein and lactose content (and other low molecular weight components), maintain the osmotic pressure of milk a constant value since a biological fluid milk must be iso-osmotic with blood. Changes in the concentration of monovalent ions, and presumably other low molecular weight osmotically active species, are compensated by opposite changes in lactose content.

The constancy of the osmotic pressure of milk results in the depression of freezing point also being constant within narrow limits. This phenomenon has been widely applied to monitor the ingress of water into milk and it is normally accepted

that, if the freezing point depression of milk is less than 0.525°C, the milk has been adulterated.

Seasonal changes in the balance between soluble and colloidal calcium are predicated by the citrate content of the milk. Citrate is an effective buffer of both calcium and hydrogen ions and, at pH values close to neutrality, citrate from a strong complex with calcium (CaCit). This is the predominant form of the soluble calcium and citrate found in milk. Any factor which changes the citrate concentration of milk, either by dietary means or as a consequence of processing, results in a change in the concentration of soluble calcium.

The total citrate content of milk is influenced by the diet of the cow and, in particular, by the effect of diet on the *de novo* synthesis of fatty acids within the mammary gland. Essentially, when *de novo* synthesis is inhibited, citrate levels rise, and when *de novo* synthesis is most active, citrate levels are low.

Although the citrate content of milk has a profound influence on the stability of some milk systems, inorganic phosphate is also an important mineral. When milk is heated, inorganic phosphate may be precipitated on the casein micelles. As a result, the level of soluble calcium is reduced, and this is associated with improvements in the stability of evaporated milk and of products recombined from dried skim milk and anhydrous butterfat.

The minerals in milk are of high nutritional value and consumption of a modest weight of dried skim milk (approx. 60 g) can provide the recommended dietary allowance (RDA) for calcium and 75% of the RDA for phosphorus. Whilst dietary calcium has been widely recognized as a key factor in determining healthy bone development in young children and in the fetal child, it is also recognized that calcium status may be factor in the development of osteoporosis in post-menopausal women. However, it is doubtful if consumption of extra calcium in later life can reverse the deterioration of bones in those susceptible to osteoporosis and the dietary supplementation by calcium may be more effective in later life when bones (and teeth) are still actively developing.

Milk is also rich in a wide range of other elements. However, only a small number, including magnesium, zinc, selenium, molybdenum and iodine, are thought to contribute significantly to the requirement for essential trace elements.

2.1.5. Minor components and micronutrients

As befits a complex biological fluid, milk contains a large number of minor components, some of which are biologically or chemically active. Urea is a minor component of milk but is responsible for almost all of the seasonal variation in the heat stability of unconcentrated milk. The concentration of milk urea is controlled by the level of urea in the blood which, in turn, is directly related to diet. The mechanism by which urea influenced heat stability is not entirely clear but most evidence point to mechanism whereby urea decomposes on heating to yield isocyanate, which then reacts with free sulphydryl groups in the whey protein and/or kappa casein. High levels of urea are associated with very stable milk.

Milk is also rich in lipoprotein lipase; an enzyme activity has been reported. However, only a small number of enzymes are of practical significance. The principal lipase in milk is lipoprotein lipase, an enzyme which catalyses the hydrolysis of triglycerides to free fatty acid. This reaction results in the production of soapy, bitter, rancid and unclean flavors in dairy products. The enzyme is present in freshly drawn milk in great excess and, under certain conditions, will spoil the milk within a few minutes. However, two factors prevent spoilage of milk by lipoprotein lipase. First, the natural milk fat globule membrane acts as a physical barrier to lipase action and, second, the lipase is readily destroyed by heat treatment. Spontaneous lipolysis is sometimes enhance in raw milk and seems to be influenced by a number of factors. These include the stage of lactation, season, diet, and plane of nutrition.

The major proteinase in milk is plasmin, a serine proteinase with trypsin-like activity. The generally held view is that plasmin is identical to the blood enzyme of the same name, and its concentration in milk is probably associated with physiological conditions where leakage of blood components into milk is favored. For example, high milk plasmin levels have been associated with milk from cows in very early lactation, in late lactation and when udder disease is present. As mentioned

earlier, high levels of gamma casein in bacteriologically sound milk are a good index of plasmin activity. At neutral pH values, milk plasmin level is very heat stable.

Lactoperoxidase is another milk enzyme which is present at high concentrations. It catalyses the transfer of oxygen from hydrogen peroxide to other substrates such as thiocyanate. Lactoperoxidase has the potential to catalyse oxidation of unsaturated fatty acids leading to the development of oxidized flavours. On the other hand, if the levels of thiocyanate and peroxide in milk are supplemented, lactoperoxidase acts as a powerful bacteriocide which can kill *coliforms, Salmonellae, Shigellae* and *Pseudomonads*. This system has been widely promoted for short-term preservation of milk in developing countries where refrigeration is scarce.

Xanthine oxidase is present in milk and can catalyze non-specific oxidation of dairy products. Nevertheless, its overall significance is unlikely to be high. Although not a degradative enzyme, alkaline phosphatase is worthy of mention since it is almost completely inactivated by pasteurization, and is therefore used as an index of the efficiency of such heat treatment.

A substantial number of vitamins are found in milk. These may be divided into the fat soluble vitamins A, D and E, and the water soluble components vitamin C, vitamin B_1, B_2, B_6, B_{12}, pantothenic acid, niacin, biotin and folic acid.

The seasonal changes in the water soluble vitamins in milk are generally small but the concentrations of some fat soluble vitamins are very dependent on the diet and, to a lesser extent, the breed of cow.

The vitamins in milk are readily affected by processing. Vitamins C, B_2 and A are all affected by light to varying degrees, and deterioration of milk exposed to bright light should be avoided. The fat soluble vitamins are relatively stable to heat but the water soluble vitamins, B_1, B_6, B_{12} and folic acid are less heat stable.

2.2 Variability *(27)*

Cow's milk is remarkably constant in a qualitative sense, but quantitative variation in composition is important. Broadly speaking, the relative variation in the milk yield of a cow is wider than that in composition. The structure of the milk (e.g., the size of the fat globules) and other properties (such as heat stability) vary as well. The widest variation usually occurs in fat content; variation in protein is less and that in ash still less.

Variation in the fatty acid pattern of milk fat is considerable. Each individual protein is of constant composition except for genetic variants and such posttranslational modifications as phosphorylation and glycosylation. The proportions of the several caseins are relatively constant. Kappa casein being the most variable. The ratio of casein to whey proteins is fairly constant, but the proportions of individual whey proteins vary more than those of the caseins. Some variation in mineral composition occurs.

The variability of milk thus depends on genetic factors, (breed, and individual) the physiological condition of the animal (stage of lactation, age of the cow, estrus and gestation) and environmental factors (feed, climate, method of milking).

2.2.1 Breed

A wide diversity of breeds of cow exists. Breeds should not be considered subspecies; they are predominantly the result of selection by people to obtain cattle suitable for the production of milk, or meat and fit for local conditions, such as climate, feed, terrain, and customs. This has led to wide variability in milk yield and, to a somewhat lesser extent, composition. Typical dairy breeds, however, do not show much variation in milk composition.

2.2.2 Individuals

Variation in milk composition among individual cows of one breed may be greater than that among breeds. Differences in composition in the milk of different quarters of the udder of one cow mostly are negligible.

2.2.3 Stage of Lactation

The time elapsed after partition or calving considerably influences milk composition, presumably because the needs of the calf change with age. To be sure, the time at which the cow becomes pregnant again also affects milk composition and the duration of lactation. It is common to milk cows to ten months and leave them dry for two months, but they can be milked right up to the next calving. Prolonging lactation after 10 months may eventually cause milk composition to become very different. Other constituents change as well, some to an even greater extent. The pH is on average at first about 6.6, then for a period about 6.7, and rises to about 6.9 at the end; this implies that H^+ activity varies by a factor of 2. In the course of lactation the total ionic strength and the Ca^+ activity decrease (especially at first), the proportion of Ca and the phosphate associated with the casein micelles increases, and the concentration of calcium phosphate in the micelles slightly decreases. The quantity of P present in soluble esters decreases from about 250 to about 50 mg/kg of milk. The ratio of caseins to whey proteins remains practically constant for many cows, but the proportions of individual whey proteins change; for instance, serum albumin increases and immunoglobulins decrease with lactation. The tendency for the fat globules to agglutinate in the cold markedly decreases with lactation. At the very end of lactation milk composition becomes slightly more similar to blood serum; the tendency to lipolysis may increase considerably. The lactation stage is the main variable, though it is difficult to separate the effect from that of other variables such as feeding regimen and grazing.

2.2.4 Colostrum

The first secretion after parturition has a very different composition and is called colostrums or beestings. Colostrum is somewhat similar to blood serum and may even contain some red blood cells. It is also high in leukocytes. The protein composition is highly specific; immunoglobulin content in the first colostrum mostly is about 7.0%, as compared to less than 0.1% in mid lactation milk. The high serum protein content causes colostrums to gel like egg white when heated at 80 °C. Cu content is greater than 100 μg/kg, as compared to 20 μg/kg in mid lactation, and Fe content also is high. Enzyme composition is markedly different, colostrums, for instance, containing greater quantities and more species of proteolytic and lipolytic enzymes. The pH can be as low as 6.0.

2.2.5 Other Physiological Factors

Estrus and gestation do not have a great effect on milk composition, but they have on milk yield. Most of the milk components decrease slightly in concentration with age of the cow, but Na increases.

2.2.6 Mastitis

Severe inflammation of the udder after pathogenic bacteria have entered causes a decrease in milk yield and a change in milk composition, and the number of somatic cells in the milk increases. Severe mastitis causes milk composition to somewhat resemble that of blood serum, just as at the very end of lactation. Also, certain enzymes increase in activity. Usually the presence of mastitis milk does not cause significant problems for the dairy manufacturer, but mastitis causes a significant loss for the farmer.

2.2.7 Feed

Environmental factors may greatly affect milk yield but have less influence on milk composition. Feed composition can affect the fat content of milk and especially its fat composition. A low-protein diet causes the protein content of the milk to

decrease somewhat, whereas a high-protein diet causes non protein N content to increase. Several minor components are strongly affected by the content in the feed.

2.2.8 Other Environmental Factors

Climate has little effect on milk composition unless it is extreme, causing heat stress. High ambient temperatures (>30°C) tend to give higher fat, lower N, and lower lactose content.

2.2.9 Milking

The shorter the time elapsed after the previous milking, the lower the milk yield and the higher the fat content will be. Hence, evening milk usually has a higher fat content than morning milk. Incomplete milking thus can decrease the fat content of a milking, although not that of the milk on average. Short time intervals between milkings increase the susceptibility of the milk to lipolysis.

2.2.10 Random Variations

Day-to-day fluctuations occur in the fat content especially. Milking routine can also affect milk composition as well as yield.

2.3 The Flavor of Milk *(28)*

Fresh milk of good quality has a bland but characteristic flavor. The sensory perception it brings about is determined mainly by the pleasant mouth feel from the physical constitution of milk, which is an emulsion of fat globules in an aqueous colloidal protein phase, and a slight salty sweet taste that results from milk salts and lactose.

An important aroma constituent of fresh milk, have been identified. These belong to many different classes, such as carbonyl compounds, alcohol, fatty acids, lactones, esters, sulfur compounds, nitrogen compounds, and aliphatic and aromatic hydrocarbons.

The odors and flavors of the separated fractions were evaluated in order to determine which compounds contribute to the overall aroma of milk. It is clear that the aroma of milk is determined by many volatile compounds mostly present in very low concentrations below their flavor thresholds. The total aroma concentrate is estimated to be 1-100 mg/kg. A number of these compounds may be transferred from the feed to the milk while others result from minor conversions of milk constituents by chemical, microbial and enzymatic, reactions. If the quantity of aroma compounds formed exceeds a certain level, the flavor balance may be upset and off-flavors result. Flavor threshold values for a number of compounds that contribute to flavors in dairy products are given in Table 2.6.

Table 2.6 Flavor threshold values of compounds contributing to (off)-flavors in milk and milk product.

Compound	Flavor threshold (mg/kg)
n-Alkanoic acids	
Butanoic acid	25
Hexanoic acid	14
Octanoic acid	10
Decanoic acid	13
Dodecanoic acid	9
n-Alkanals	
Ethanal	1.50
Propanal	0.50
Butanal	0.20
Pentanal	0.15
Hexanal	0.07

Table 2.6 (continued).

Compound	Flavor threshold (mg/kg)
n-Alkanals	
Heptanal	0.10
Octanal	0.10
Nonanal	0.20
Decanal	0.30
n-Alkanols	
Butanol	0.5
Pentanol	0.4
Hexanol	0.5
Heptanol	0.6
Lactones	
γ10	1.5
δ10	2.0
2-Alkanones	
2-Butanone	80.0
2-Pentanone	9.0
2-Hexanone	0.4
2-Heptanone	0.7
2-Octanone	0.5
2-Nonanone	4.0
2-Decanone	15.0
2-Undecanone	20.0
Ethyl esters	
Ethyl butanoate	0.03
Ethyl hexanoate	0.08

Table 2.6 (continued).

Compound	Flavor threshold (mg/kg)
Miscellaneous	
1-Octen-3-one	10.0[a]
Diacetyl	20.0[a]
Dimethyl sulfide	20.0[a]

[a] *Value expressed as μg/kg*

2.3.1 The Origin of Flavor Compounds in Milk

2.3.1.1 Flavor Compounds in Freshly Collected Milk

Most flavor compounds present in normal fresh milk (carbonyl compounds, alkanols, free fatty acids, sulfur compounds, etc.) are probably produced through the cow's metabolism, even when the feed is an odorless synthetic diet. However, volatiles may also be transferred from the forage to the milk via the rumen (and the respiratory tract).

2.3.1.2 Flavor Compounds Originating from Milk Fat

Milk fat is an important source of flavors and off-flavors in milk.

Free fatty acids are released from glycerides by the action of milk lipase or bacterial lipases, particularly from psychrotrophic bacteria 2-Alkanones (odd-numbered C_3-C_{15}) are formed by thermal decarboxylation of β-keto acids.

4-and 5-Alkanolides (mainly even-numbered C_6-C_{16} 4-alkanolides) are formed from the corresponding 4-and 5-hydroxy fatty acids by intramolecular esterification, usually under the influence of heat.

Auto-oxidation products are formed from unsaturated fatty acids by nonenzymatic autocatalytic oxidation reactions resulting in the formation of hydroperoxides. These hydroperoxides may dismutate to secondary oxidation

products such as aldehydes and ketones. The latter group contains many important flavor compounds with specific aromas and usually very low flavor threshold values.

2.3.1.3 Flavor Compounds Originating from Proteins, Lactose, and Thiamin

Proteins and lactose can be important sources for the formation of flavor compounds in milk by chemical, enzymatic, and microbial processes.

Sulfur compounds can be formed by heat from β-lactoglobulin and fat-globule membrane material. Methanethiol, dimethyl disulfide, and methional can be formed from methionine when milk is exposed to light.

Heterocyclic compounds such as pyrazines, pyrroles, pyridines, thiazoles, furand, and maltol are produced by nonenzymatic browning reactions.

Peptides with a very bitter taste can be formed from milk proteins by the action of milk proteinases or bacterial proteinases. The latter enzymes in particular may cause problems because of their very high thermostability.

Photochemical degradation of thiamin hydrochloride (vitamin B_1) followed by reaction with H_2S produces a compound with a rubbery or boiled milk odor and an extremely low odor threshold value.

2.3.2 Off-Flavors in Milk

2.3.2.1 Off-Flavors Caused by Extraneous Components

a) Absorbed Flavors. Fresh milk may have off-flavors from the transfer of certain components from the feed to the milk. There are two pathways by which flavor compounds may enter the milk through the cow. One is by the pulmonary tract to the bloodstream, to the mammary cells, and into the milk. The other is through the digestive tract to the blood and thence into the milk. It should be noted that odors in a stable (from the feed, from contaminated air, or from substances given off by the cow in the breath, sweat, urine, and feces) may accumulate readily in the milk by the inhalation route and can lead to off-flavors. Also, residual disinfectants may impart

off-flavors to milk. The direct transfer of odors by exposing milk to the air in the stable seems to be less significant.

b) Feed Off-Flavors and Weed Taints. Odor and flavor are characteristic of the associated feed; silage, hay, grassy, etc. Can be slightly sweet and generally is not unpleasant though may be perceived as unclean when extreme or when feed quality is poor. Most feeds defects clear up readily after discharge from mouth.

These are caused by increased quantities of benzyl mercaptan, methyl mercaptan, dimethyl sulfide, dimethyl disulfide, indole, skatole, and trimethylamine.

c) Cowy Flavor. Cowy flavor sometimes occurs in raw milk, especially in the winter period: It can be attributed in part to the transfer of taints from the air in the stable or from the feed. However, it seems to be related particularly to ketosis or acetonemia, a disease in cattle involving the endogenous energy metabolism and leading to increased concentrations of acetone in milk.

d). Oxidation Flavor Defects. (Metallic-oxidized) An important source of flavor defects in milk and dairy products is oxidation of the lipid fraction. These defects are caused chiefly by increased concentrations of carbonyl compounds resulting from the oxidative breakdown of unsaturated fatty acids by copper or certain other metals contaminating milk (i.e. copper pipe, white metal). May be associated with excess of certain high fat feeds (i.e. soybeans) and/or lack of antioxidants (i.e. vitamin E) in feed rations.

e) Heat Induced Flavors. These flavors are a separate subject. Mostly, they are the consequence of a thermal treatment to which milk and its products must be subjected in order to obtain sufficient keeping quality milk lipids are another source of heating flavors. Methyl ketones (2-alkanones) are formed upon thermal decarboxylation of β-keto fatty acids (liberated by hydrolysis of milk fat). Similarly, γ- and ε-hydroxy fatty acids may produce the corresponding lactones

f) Light Induced Flavor. Light induced flavor can occur in milk and milk products upon exposure to (sun) light, resulting in protein degradation and/or lipid oxidation.

2.3.2.2 Off-Flavors Caused by Microorganisms and Enzymes

The growth of microorganisms in milk and dairy products easily may produce different kinds of flavor defects. Similar defects may be caused by the action of bacterial or milk enzymes.

a) Unclean flavor. Unclean flavor may be the result of an increase in the concentration of dimethyl sulfide, as caused by rod-shaped gram-negative psychotropic bacteria, which may develop at temperatures of 4°C or above.

b) Fruity Flavor. Fruity flavor results from ethyl esters of butyric, isovaleric, and caproic acid, which are produced by certain psychotropic organisms such as *Pseudomonas fragii.*

c) Malty Flavor. Malty flavor is caused mainly by 3-methyl butanal, 2- methyl butanal, and 2-methyl propanal, which result from growth of *Streptococcus lactis* var. *maltigene* due to poor refrigeration.

d) Phenolic Flavor. Phenotic flavor may develop occasionally in in-bottle sterilized milk because of contamination with spores of certain types of *Bacillus circulans.* Cresols probably are responsible for this defect.

e) Bitter Flavor. Bitter flavor occurs occasionally in UHT milk. This defect is caused by the breakdown of milk proteins by proteinases during prolonged storage to short latter peptide. A sufficiently intensive heat treatment (more than 16 s at 142°C) may inactivate the milk proteinase (plasmin), but some bacterial proteinases will resist even very rigorous heat treatment (6 min at 142°C).

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f) Rancid Flavor. Rancid flavor in milk and milk products is caused by liberation of lower fatty acids (C_4-C_{12}) by milk lipase or by bacterial lipases. In raw milk it is associated with excessive agitation, temperature abuse, or cow related factors (i.e. health, nutrition).

It should be emphasized that rancidity in milk as well as unclean and fruity flavors are important problems in connection with present methods of bulk milk collection and prolonged storage of raw milk.

2.4 Sample Preparation

A Key Step in Chemical Analysis of Dairy Foods is good sample preparation. It is usually not possible to directly inject a food sample into a GC without performing some sample preparation. Proteins, fats, complex carbohydrates and other nonvolatile chemicals will degrade in the heated GC injector, resulting in the formation of numerous artifact peaks that can degrade column performance and obscure peaks of interest. Separating volatile compounds from matrix interferences and concentrating volatiles (which can be present in concentrations as low as 10^{-8} to 10^{-14} %) so they can be detected usually requires steps of sample preparation involving volatile isolation and concentration steps.

Unfortunately, there is no one perfect sample preparation technique to use for flavor research. The aroma volatiles in food samples can be heterogeneous, covering a wide range of polarity, solubility, functional groups, vapor pressures, concentrations and volatilities. Other complications include instability of aroma volatiles to certain conditions (oxygen, light, heat, pH, etc.) and the possibility that aroma volatiles may interact with chemicals in the food matrix. It is important that the extraction technique does not introduce or create volatiles that are not in the dairy product being tested. For example, sample preparation techniques that involve heating the sample to high temperatures (e.g., steam distillation) can generate artifact peaks in sample chromatograms, and these odoriferous artifacts may be misinterpreted as the cause of the off-flavor problem in the product. In some cases, more than one procedure may be required for optimum recovery of flavor compounds.

Dairy chemists now have a wide variety of sample preparation techniques that they can use for isolating and concentrating odor-active chemicals prior to GC analysis. Frequently used sample preparation methods for flavor analysis include vacuum distillation, simultaneous steam distillation/extraction (also referred to as the Liken and Nickerson extraction procedure), static headspace, dynamic headspace and solid-phase microextraction (SPME). Some of the more popular sample preparation techniques for flavor analysis are discussed below.

2.4.1 Solvent extraction and distillation *(30)*

Solvent extraction commonly involves the use of pentane, dichloromethane, diethyl ether or some other volatile organic solvent. This limits the method to the isolation of fat-free foods unless an additional procedure is employed to separate the extracted fat. Some researchers have performed solvent extraction of aroma volatiles from aqueous distillates prepared by low temperature/high vacuum distillation. Figure 2.2 show one of the propose designs for the simultaneous distillation and extraction apparatus.

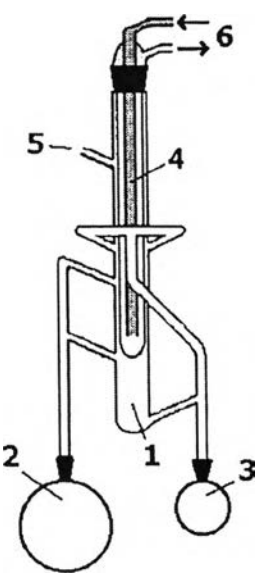

Figure 2.2 Likens-Nikerson simultaneous distillation extraction apparatus
(1. body; 2. sample flask; 3. extracting solvent flask; 4. cold tube;
5. inlet for purge gas; 6. cold water inlet and outlet)

W. Engel et al. developed a new distillation unit, called solvent assisted flavor evaporation (SAFE), for the extraction of flavor volatiles from complex aqueous matrices, such as beer, fruit juices, milk and cheese. The distillation vessel and "transfer tubes" are thermostated at low temperatures (20°C–30°C) to avoid condensation of compounds with high boiling points, and the sample is added by dropping aliquots from the funnel into the vessel to reduce time of extraction. This new method allows for the use of solvents other than diethyl ether and dichloromethane, and it could be used for extracts containing large concentrations of fat.

Solvent extraction methods have disadvantages. Large volumes of solvent must be evaporated while retaining the volatile flavor components. Another problem is that sample preparation is time consuming; only one or two samples can be extracted per day.

2.4.2 Static Headspace Extraction *(30)*

Static headspace extraction is also known as equilibrium headspace extraction or simply as headspace. It is one of the most common techniques for the quantitative and qualitative analysis of volatile organic compounds. With the current availability of computer-controlled instrumentation, automated analysis with accurate control of all instrument parameters has become routine.

If a complex material, such as milk, yogurt or cheese, is placed in a sealed vessel, some of the more volatile compounds in the sample matrix will leave the sample and pass into the headspace around it. If the concentration of the volatile compound reaches about 1 ppm in the headspace, it may be assayed by a simple injection of an aliquot of the atmosphere in the vessel. How much compound enters the headspace depends on several factors, including its concentration in the original sample, the volatility of the chemical, the solubility of the chemical in the sample matrix, the temperature of the vessel and how long the sample has been inside the vessel.

In practice, the food sample is placed into a headspace vial, sealed and warmed to enhance vaporization of the volatiles and incubated for a period of time to establish equilibrium at the incubation temperature. Once the volatiles have equilibrated, an aliquot of the headspace gases is withdrawn with a syringe and injected into the GC. As an alternative, the equilibrated headspace may also be allowed to pass through a sample loop of known volume, which is subsequently flushed into the injection port.

Static headspace methods eliminate the large solvent peak, which may obscure important odor-active analytes. Static headspace is a relatively rapid technique that is easily automated, making it attractive for sample screening applications. The combination of careful monitoring of temperature and equilibrium time, pressure control of the sample loop and automatic injection provides increased reproducibility over manual attempts at headspace analysis and reduces labor costs. Additional advantages include low cost per analysis, simple sample preparation and the elimination of reagents.

Relatively poor sensitivity compared to other types of sample preparation techniques is a disadvantage of static headspace methods. The maximum temperature for most food products is less than the boiling point of water. Analysis at this fairly low temperature limits the usefulness of the technique for analytes with boiling points over approximately 130°C. Many materials that may be extracted with solvents may elute well at higher GC column temperatures but will be poorly represented in a static heads pace chromatogram. Also, reproducibility depends on analyzing a sample after it has reached equilibration, and the time required to achieve this point may, especially for less volatile compounds, be a drawback for some analyses.

2.4.3 Dynamic Headspace Extraction (Purge and Trap) *(30)*

For the analysis of trace quantities of analytes, or where an exhaustive extraction of the analytes is required, purge and trap, or dynamic headspace extraction, is preferred over static headspace extraction. Like static head-space sampling, purge and trap relies on the volatility of the analytes to achieve extraction from the matrix. However, the volatile analytes do not equilibrate between the gas phase and matrix.

Instead, they are removed from the sample continuously by a flowing gas. Consists of a purge vessel, a sorbent trap, a six-port valve, and transfer lines. Instrument of purge and trap is shown in Figure 2.3.

A purge and trap cycle consists of several steps: purge, dry purge, desorb preheat, desorb, and trap bake. First, a sample is introduced into the purge vessel. Then the valve is set to the purge position such. During purge, dry purge, and preheat, desorb (carrier) gas directly enters the GC. Typically, the purge time is 10 to 15 minutes, and the helium flow rate is 40 mL/min. The trap is at the ambient temperature. After purging, the purge gas is directed into the trap without going through the sample, called dry purge. The purpose of dry purging is to remove the water that has accumulated on the trap. Then the purge gas is turned off, and the trap is heated to about 5 to 10°C below the desorption temperature. Preheat makes the subsequent desorption faster. Once the preheat temperature is reached, the six-port valve is rotated to the desorb position to initiate the desorption step. The trap is heated to 180 to 250°C and back-flushed with the GC carrier gas. The flow rate of the desorb gas should be selected in accordance with the type of GC column used. After desorption, the valve is returned back to the purge position. The trap is reconditioned/baked at (or 15°C above) the desorption temperature. The purpose of trap baking is to remove possible contamination and eliminate sample carryover. After baking, the trap is cooled, and the next sample can be analyzed.

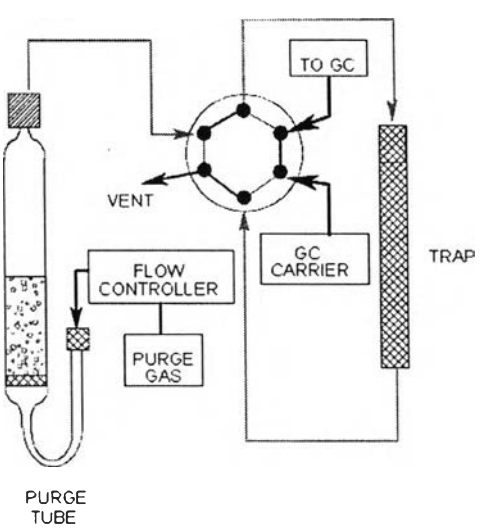

Figure 2.3 Instrument of purge and trap *(30)*

Dynamic headspace is significantly more sensitive than static headspace. Compared to solvent extraction techniques, it offers the advantages of no solvent to evaporate, no interfering solvent peaks in chromatograms and relatively simple automated sample preparation.

The disadvantages include more complicated instrumentation. Instrumentation must monitor several steps, valving, heated zones, etc. Instrumentation is more expensive than static headspace instrumentation. Because of complex functioning of the instrument, there are many opportunities for malfunction, including heater damage, valve leaking, contamination and cold spots. Compared to static headspace, dynamic headspace techniques require a little more time per sample (for purging, trap drying and trap transfer, all of which typically require approximately 15 min). However, the technique is much faster than most solvent extraction techniques.

2.4.4 Solid Phase Microextraction (SPME)

Solid-phase microextraction (SPME) is a relatively new method of sample introduction, developed by pawliszyn and co-workers in 1989 and made commercially available in 1993. SPME is a solventless extraction method that employs a fused silica fiber coated with a thin film of sorbent, to extract volatile analytes from a sample matrix. The fiber is housed within a syringe needle that protects the fiber and allows for easy penetration of sample and GC vial septa.

SPME has several advantages in the analysis of volatile organics. First, no additional instruments or hardware are required. Second, the cost of fibers is low compared to the cost of other methods for volatile analyte extraction. Fibers can be reused from several to thousands of times, depending on extraction and desorption conditions. SPME requires minimal training to get started, although there may be many variables involved in a full-method development and validation. SPME is also easily portable, and field sampling devices are readily available. Finally, with a variety of fiber coating chemistries available, SPME can be applied to a wide variety of volatile organic analytes.

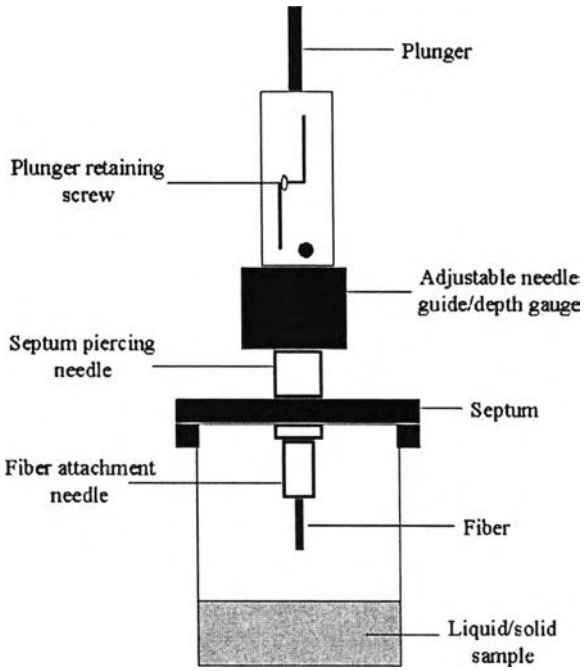

Figure 2.4 Instrument of classical headspace sampling.

There are two approaches to SPME sampling of volatile organics: direct and headspace. In direct sampling the fiber is placed directly into the sample matrix, and in headspace sampling the fiber is placed in the headspace of the sample. Instrument of classical headspace sampling shown in Figure 2.4. In the Figure 2.5, Solid phase microextraction procedure is showed.

For dairy products, which contain high levels of fat, carbohydrate and protein, the headspace technique is preferred.

SPME fibers have different coatings with different polarities are used on SPME fibers. Currently, three classes of fiber polarity coatings are commercially available: nonpolar, semipolar, and polar coatings. There are several advantages of using different fiber polarities. For one, using a matched-polarity fiber (i.e., polar-coated for a polar analyte) offers enhanced selectivity. Also, there is less of a chance of extracting interfering compounds along with the analyte of interest. And an organic matrix is not problem-polar compounds can still be extracted.

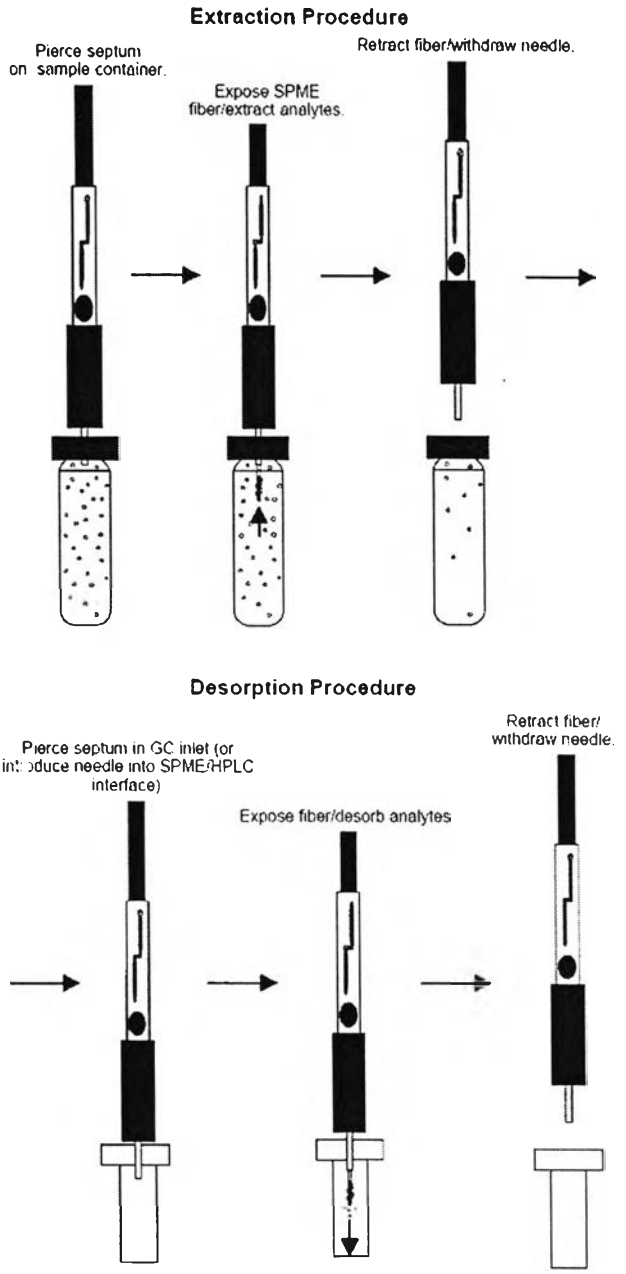

Figure 2.5 Solid Phase Microextraction procedure.

Once the fiber is chosen, extraction conditions must be optimized. Extraction time is optimized by extracting a standard using a range of extraction times and plotting the analyte GC peak area versus the extraction time. As extraction time is increased, a plateau in peak area is reached. This represents the time required for the system to reach equilibrium and is the optimized extraction time. If the extraction time can be controlled carefully and if sensitivity is adequate, shorter extraction time can be used without fully reaching equilibrium.

The sample volume also has an effect on both the rate and recovery in SPME extraction, as determined by extraction kinetics and by analyte partition coefficients. For this reason, in very dilute samples, larger sample volume results in slower kinetics and higher analyte recovery.

As with any extraction, the agitation method will affect both the extraction time and recovery and should be controlled as closely as is practical. Typically, headspace SPME sample vials are not agitated.

Extraction temperature can also be an important factor, especially in headspace SPME analyses. However, in SPME, unlike in GC headspace analysis, increasing the temperature in SPME can result in a maximum usable temperature for the method (i.e., going from 25°C to 30°C may result in a reduction in sensitivity.

The sample matrix may also be modified to enhance extraction recovery. This is typically done by either dissolving a solid sample in a suitable solvent, usually water or a strongly aqueous mixture, or by modifying the pH or salt content of a solution.

SPME is particularly well-suited to the analysis of dairy products. The technique is capable of extracting a broader range of analytes than is possible with other headspace techniques. For example, SPME is capable of ppb detection levels for both low molecular weight, highly volatile compounds like acetaldehyde, dimethyl sulfide, acetone and 1,3-pentadiene, as well as high molecular weight, high-boiling-point compounds like vanillin, lactones and dodecyl aldehyde. Furthermore, it can be used for quantitating free fatty acids (C4 through C14) in dairy products. This important class of flavor compounds can be particularly challenging and time consuming to extract by other techniques.