CHAPTER 5

Chemical and Instrumental Approaches to Cheese Analysis

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Contents

Abstract

Overcoming the complexity of cheese matrix to reliably analyze cheese composition, flavor, and ripening changes has been a challenge. Several sample isolation or fractionation methods, chemical and enzymatic assays, and instrumental methods have been developed over the decades. While some of the methods are well

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established standard methods, some still need to be researched and improved. This chapter reviews the chemical and instrumental methods available to determine cheese composition and monitor biochemical events (e.g., glycolysis, lipolysis, and proteolysis) during cheese ripening that lead to the formation of cheese flavor. Chemical and enzymatic methods available for analysis of cheese composition (fat, protein, lactose, salt, nitrogen content, moisture, etc.) are presented. Electrophoretic, chromatographic, and spectroscopic techniques are also reviewed in the light of their application to monitor cheese ripening and flavor compounds. Novel instrumental methods based on Fourier-transform infrared spectroscopy that are currently being researched and applied to cheese analysis are introduced.

I. INTRODUCTION

Cheese analyses include compositional analysis, microbiological evaluations, and analysis of ripening products. Determination of overall quality, evaluation of manufacturing process, and cheese research studies require analysis of the breakdown products of ripening, in addition to compositional analysis. Several chemical methods have been developed and modified over the years. Studies on cheese ripening and flavor analysis greatly benefited from the development of electrophoresis and chromatography. This chapter presents a review of chemical and instrumental methods available for cheese analysis. Several of these methods have also been reviewed by many books (Fox, 1999; Fox *et al.*, 2004a,b; McSweeney and Fox, 1993; Nielsen, 2003; Wehr and Frank, 2004) and review articles (McSweeney and Fox, 1997; Singh *et al.*, 2003). In addition, this chapter introduces novel, rapid, simple, and cost-effective applications based on infrared spectroscopy that have been developed or applied to cheese analysis.

II. SAMPLING TECHNIQUES

Irrespective of the type of analysis, sampling of cheese requires extensive care and thought. The International Organization for Standardization (ISO), Association of Official Analytical Chemists (AOAC), International Dairy Federation (IDF), and some authors have published techniques, guidelines, and requirements for sampling cheese (AOAC, 2005; Bintsis *et al.*, 2008; Fox *et al.*, 2004a; Grace *et al.*, 1993; IDF, 1995; ISO, 2008a). In general, cheese may be sampled by (a) cutting, (b) using a trier, or (c) taking the entire cheese. Cutting involves making two radiating cuts from the center of the cheese (for cheeses with circular base) or parallel to the sides (for cheeses with rectangular base) to sample at least 50 g of cheese. Sampling using a cheese

trier depends on the shape, weight, and type of cheese and may be done by inserting the trier (1) obliquely toward the center of the cheese, (2) perpendicularly into one face and passing through the center to reach the opposite face, (3) horizontally into the vertical face of cheese, halfway between the two plane surfaces, toward the center of the cheese, or (4) obliquely through the contents of the container from the top to the base (in the case of barrels or large blocks of cheese).

Plugs of depth 15–20 mm should be collected from various surface and subsurface (for research studies) locations using a clean and sterile (for microbiological or sensory samples) trier. The sampling location depends on the shape of the cheese. A pictorial representation of suggested sampling techniques based on various shapes and sizes (cylinder, cube, block, sphere, etc.) was presented by Fox *et al.* (2004a). To collect laboratory samples from very large barrels, the AOAC (2005; standard 970.30) suggests using a 12-in. trier to sample a plug at least 10 in. long. The trier must be inserted into the cheese at about 7 cm from the edge of the cheese and at an angle of 11°. A trier guide may be used as an aid. The top 4.5 in. section of the plug is used to reseal the plug hole, the next 4 in. is used for analysis and any remaining length of sample is discarded. For reliable analysis, duplicate samples (at least 100 g) should be taken at refrigerated temperature or colder and analyzed as soon as possible within 24 h. In the case of cheese with a composition gradient, brine-salted cheese or cheese with surface microflora (such as mold-ripened cheeses), sampling by cutting is recommended instead of a trier. Sampling the entire cheese is generally done for smaller portioned cheeses. All samples should be accompanied by a sampling report containing place, date, and time of sampling, details of the sampling person, method of sampling, batch/lot number of the samples (or any identification details that would ensure traceability), place to which samples are being sent to, and comments on environmental/storage conditions.

III. COMPOSITIONAL ANALYSIS

Analysis of cheese composition, including moisture, protein, fat, ash, and salt, is usually conducted according to the methods published by ISO, IDF, or AOAC. Some of the standard methods available for cheese analysis are summarized in Table 5.1 and briefly discussed below. Additional information may be found elsewhere (Nielsen, 2003; Nollet, 2004).

A. Moisture and total solids

Moisture and total solids in cheese are usually determined by gravimetric methods. The most common oven drying method (AOAC 948.12, 2005; ISO, 2004a) involves drying the sample in the conventional oven at 102 °C

TABLE 5.1 Standard methods for compositional analysis of cheese

Constituent	Method[a]	Principle
Moisture/total solids	ISO 5534|IDF 4 (ISO, 2004a) AOAC 948.12 (2005)	Oven drying at 102 °C
	AOAC 926.08 (2005)	Vacuum oven drying
	AOAC 969.19 (2005)	Distillation
	AOAC 977.11 (2005)	Microwave oven drying
	ISO 2920|IDF 58 (ISO, 2004b)	Oven drying at 88 °C (for whey cheeses prepared by concentration)
Fat	ISO 1735|IDF 5 (ISO, 2004c)	Gravimetric solvent extraction (Schmid–Bondzynski–Ratzlaff) method
	AOAC 933.05 (2005)	Babcock method
	ISO 8262-3|IDF 124-3 (ISO, 2005)	Gravimetric Weibull–Berntrop method for fresh cheese types
	ISO 1854 (ISO, 2008b) IDF 59A (IDF, 1986)	Gravimetric Röse–Gottlieb method
Protein (total nitrogen)	ISO 8968-1|IDF 20-1 (ISO, 2001a) AOAC 920.123 (2005)	Titrimetric Kjeldahl method
	AOAC 991.20-23 (2005)	For unripened/fresh cheese
Ash	AOAC 935.42 (2005)	Gravimetric (ashing at 550 °C)
Phosphorus	ISO/TS 2963|IDF/RM 34 (2006b)	Enzymatic spectrophotometric method (molybdate–ascorbic acid)
Chloride	ISO 5943|IDF 88 (2006a) AOAC 983.14 (2005)	Potentiometry (determination of chloride, expressed as sodium chloride)
	AOAC 935.43 (2005)	Volhard's titration method
Salt (NaCl)	AOAC 975.20 (2005)	Quantab® strip chloride titration method (based on Mohr's titration)
Acidity	AOAC 920.124 (2005)	Titration with NaOH

[a] The format "ISO XXXX|IDF YYY" denotes joint ISO–IDF standards published by ISO.

to a constant weight and calculating the moisture using the reduction in weight from drying. Recently, Bradley and Vanderwarn (2001) suggested improvements to the equipment used in the AOAC methods. Use of blenders over hand grater, rotary grater, etc. and aluminum pans with glass fiber covers over AOAC pans with insert cover and filter paper resulted in a smaller standard deviation. For whey cheeses prepared by concentration, drying is done at 88 °C (ISO, 2004b). Drying can also be done in a vacuum oven (AOAC 926.08, 2005) or a microwave oven (AOAC 977.11, 2005). Certain soft cheeses or mold-ripened cheese varieties, which are high in substances other than water (e.g., volatile fatty acids), may be analyzed by distillation (AOAC 169.19, 2005) with an immiscible solvent (e.g., *n*-amyl alcohol and xylene, 1:2). The water is collected and measured in a graduated tube.

B. Fat

There are several methods available for the analysis of fat content of cheese, which to a certain extent are dependent on the type of sample. The standard method is the Schmid–Bondzynski–Ratzlaff (SBR) technique (ISO, 2004c). In this gravimetric method, a known amount of the sample is first digested with HCl and then extracted using a mixture of diethyl ether and light petroleum or alcohol. The solvent is evaporated and the remaining fat is weighed to calculate the fat content. The butyrometric Gerber or Babcock method involves digestion of the sample with H_2SO_4, followed by centrifugation and addition of hot water. The height of the fat layer in the graduated tube is measured and expressed as percent fat. In the Weibull–Berntrop method (ISO, 2005), applied to fresh cheese types such as cottage cheese and quarg, the sample is digested with HCl and filtered. The fat retained in the filter paper is extracted using a solvent such as *n*-hexane. Solvent is then evaporated and the weight of the extracted fat is determined to calculate fat content. The Röse–Gottlieb method (IDF, 1986; ISO, 2008b) uses alcohol and ammonia to precipitate and remove protein. Fat is extracted into a mixture of diethyl ether and petroleum ether. The amount of fat is gravimetrically measured after evaporating the solvent.

C. Protein

Protein content is calculated from the total nitrogen content determined by the Kjeldahl method (AOAC 920.123, 2005; ISO, 2001a). Sample is first digested with concentrated H_2SO_4 in the presence of potassium sulfate and copper sulfate catalyst to oxidize nitrogen to ammonium sulfate and then mixed with sodium hydroxide. The alkaline digest is titrated against HCl. The amount of HCl consumed is used to calculate the total nitrogen.

Multiplying the total nitrogen content by the Kjeldahl conversion factor of 6.38 yields the protein content.

D. Ash

Ash, the inorganic material remaining after combustion of organic constituents, is determined gravimetrically by the AOAC method (AOAC 935.42, 2005). A known amount of the sample is taken in a crucible and heated to 525 ± 25 °C in a muffle furnace until it is completely ashed. The ash content is calculated based on the final weight of the ash.

E. Salt and chloride content

Salt and chloride content of cheese can be determined by potentiometric methods (AOAC 983.14, 2005; ISO, 2006a), Mohr or Volhard titration (AOAC 935.43, 2005), and indicator test strips (AOAC 975.20, 2005). All of the methods involve titration with silver nitrate ($AgNO_3$), with potentiometric or colorimetric determination of the endpoint. Potentiometric determination of chloride (expressed as sodium chloride), reported by Fox (1963), involves digesting the sample with HNO_3 and titrating against standardized $AgNO_3$ until a difference of +255 mV is obtained between the electrodes. The Mohr titration method is a forward titration method involving titration of chloride ion with silver from $AgNO_3$ in the presence of K_2CrO_4. When all chloride ions have reacted, excess silver forms orange-colored Ag_2CrO_4 precipitate, indicating the endpoint. The Volhard method for analysis of salt in cheese (AOAC 935.43, 2005) is an indirect titration method in which excess $AgNO_3$ is first added to the sample to form a precipitate of AgCl. Excess silver is then titrated against standardized KSCN or NH_4SCN with ferric ion as the indicator. After all silver ions have reacted to form AgSCN, ferric ion reacts with thiocyanate to form a dark red complex (endpoint). The Volhard method is suitable for samples with acidic pH, while the Mohr method works well for samples with neutral or basic pH. Both methods require boiled water free of carbonates. The AOAC 975.20 (2005) method is an adaptation of the Quantab® strip chloride titration method, which is based on the principles of Mohr titration. This method is rapid and accurate (±10% error within a range of 0.3–10% NaCl in food).

F. Acidity and pH

The titratable acidity, which measures the total hydrogen ion concentration (both dissociated and undissociated), is more relevant to the flavor than pH. In the AOAC (2005; standard 920.124) method for measuring titratable acidity, the sample is grated and mixed with lukewarm (40 °C)

water and filtered. The filtrate is titrated against 0.1 M NaOH with phenolphthalein as the indicator. The titratable acidity of cheese is expressed as percent lactic acid. Although titratable acidity may be used as an index of ripening, it is not a suitable measure for mature bacterial-cheeses or mold- and smear-ripened cheeses, because the acidity of those cheeses increases during ripening (Fox *et al.*, 2004a).

The measurement of pH in cheese making is extremely important to control fermentation/acid production and hence the final quality. While there are no standard methods available for measuring cheese pH, there have been few methods reported in the literature. One method involves preparing a slurry of 10 g of grated cheese in water and measuring the pH potentiometrically (Fox *et al.*, 2004a). However, this method may alter the balance between colloidal and soluble calcium phosphate and hence it is preferable to measure the pH of the cheese directly. The quinhydrone electrode method (Marshall, 1992) measures the pH directly. The potential (mV) created by a paste of cheese and quinhydrone in saturated KCl is measured and used to determine the pH at a particular temperature.

G. Calcium and phosphorus

Calcium and phosphorus influence curd buffering properties and ripening characteristics. The calcium content in cheese may be determined by (1) titration with EDTA and ammonium purpurate (murexide) indicator (2) gravimetrically by precipitating calcium as calcium oxalate and weighing, or (3) atomic absorption spectrophotometry. The standard method for measuring phosphorus is the IDF (1987; standard 33C) or ISO (1984; standard 2962), which is a colorimetric assay with molybdate–ascorbate reagents or molybdovanadate. In addition, modified EDTA complexometric methods for calcium and phosphorus analysis in cheese have also been published (Kindstedt and Kosikowski, 1985).

IV. MONITORING CHEESE RIPENING

The complexity of the ripening process has attracted the attention of several researchers and numerous books and reviews have been published summarizing the current knowledge on cheese ripening (Collins *et al.*, 2003, 2004; Curtin and McSweeney, 2004; Fox, 1989; Fox and Wallace, 1997; Fox *et al.*, 1990, 2004a; Marilley and Casey, 2004; McSweeney, 2004; McSweeney and Fox, 2004; McSweeney and Sousa, 2000; Upadhyay *et al.*, 2004; Yvon and Rijnen, 2001). Biochemical changes in cheese during ripening can generally be grouped into two: (1) primary events consisting of metabolism of residual lactose, lactate and citrate, lipolysis, and proteolysis and (2) secondary events including metabolism

of fatty acids and amino acids, leading to the formation of smaller flavor compounds (McSweeney, 2004). Investigation of biochemical changes during ripening and understanding the ripening process requires precise monitoring of chemical reactions and products. Detailed reviews on methods to monitor cheese ripening are also available from Collins *et al.* (2004), Le Quéré (2004), Fox *et al.* (2004a), Upadhyay *et al.* (2004), Singh *et al.* (2003), and McSweeney and Fox (1993, 1997).

A. Assessment of lactose, lactate, and citrate metabolism

During cheese production lactose is converted to lactic acid by starter lactic acid bacteria (LAB). Any unfermented lactose is converted to D- and L-lactate by nonstarter lactic acid bacteria (NSLAB) and racemization, respectively. Lactate can be oxidized by LAB in cheese to acetate, ethanol, formic acid, and carbon dioxide at a rate dependent on oxygen availability (McSweeney, 2004). Other pathways include conversion to propionate, acetate, water, and carbon dioxide by *Propionibacterium* spp.; carbon dioxide and water by *Penicillium* spp. yeasts; and butyric acid and hydrogen by *Clostridium* spp. The rate of lactose metabolism influences proteolysis and flavor formation (Creamer *et al.*, 1985; Fox *et al.*, 1990).

Lactose and most of the products of lactose metabolism are quantified by colorimetric–enzymatic detection methods. A list of methods published on analysis of products of lactose metabolism are summarized in Table 5.2. A very common enzymatic method of lactose determination is using the Boehringer Mannheim kit (AOAC 984.15, 2005; Boehringer Mannheim, 1986; Kleyn, 1985). In this method, lactose is hydrolyzed by β-galactosidase (lactase) to D-glucose and D-galactose. The galactose is oxidized by galactose dehydrogenase to galactonic acid with the conversion of NAD^+ to NADH. The NADH formed during this reaction is stoichiometrically equivalent to the amount of lactose and is measured spectrophotometrically at 334, 340, or 365 nm (AOAC 984.15, 2005; Boehringer Mannheim, 1986; Kleyn, 1985). Glucose is phosphorylated to glucose-6-phosphate (by hexokinase and ATP), which is then oxidized by $NADP^+$ in the presence of glucose-6-phosphate dehydrogenase. The NADPH formed during this reaction is quantified spectrophotometrically and stoichiometrically related to lactose concentration (Boehringer Mannheim, 1986). Another enzymatic microassay method involves conversion of D-glucose to D-glucono-1-4-lactone and H_2O_2 by glucose oxidase in the presence of oxygen (Blais and Vailhen, 1995). The H_2O_2 is reacted with iodide in the presence of a molybdate catalyst. Iodine then reacts with polyvinyl alcohol to form a colored complex, which is measured and related to lactose content.

Boehringer Mannheim kits are also available for acids such as lactate, acetate, and citrate. In lactate analysis, lactate is oxidized by NAD^+ in the

TABLE 5.2 Some published methods for analysis of products of lactose metabolism

Compound	Method	References
Sugars (lactose, glucose, and galactose)	Enzymatic	AOAC 984.15 (2005), Kleyn (1985), Boehringer Mannheim (1986), and Blais and Vailhen (1995)
	Liquid chromatography	Zeppa *et al.* (2001), Lues *et al.* (1998), Mullin and Emmons (1997), and Bouzas *et al.* (1991)
	Gas chromatography	Harvey *et al.* (1981)
	Capillary electrophoresis	Izco *et al.* (2002)
Acids (lactate, acetate, citrate, pyruvate, propionate, etc.)	Enzymatic	ISO/TS 2963|IDF/RM 34 (ISO, 2006b), AOAC 976.15 (2005), AOAC 920.126 (2005), Boehringer Mannheim (1986), and Marier and Boulet (1958)
	Liquid chromatography	Zeppa *et al.* (2001), Dinkci *et al.* (2007), Lues *et al.* (1998), Mullin and Emmons (1997), and Bouzas *et al.* (1991)
	Capillary electrophoresis	Izco *et al.* (2002)
	Gas chromatography	Fernández-Garcia (1996) and Harvey *et al.* (1981)
Diacetyl and acetoin	Colorimetric	Walsh and Cogan (1974a,b)
	Liquid chromatography	Zeppa *et al.* (2001), Lues *et al.* (1998), Mullin and Emmons (1997), and Bouzas *et al.* (1991)
	Gas chromatography	Fernández-García (1996)
	Capillary electrophoresis	Izco *et al.* (2002)
2,3-Butanediol	Gas chromatography	Fernández-Garcia (1996)
Acetolactate	Colorimetric	Mohr *et al.* (1997)
	Gas chromatography	Richelieu *et al.* (1997)

presence of lactate dehydrogenase to pyruvate and NADH. L-Glutamate depletes pyruvate by converting it to alanine in the presence of alanine transaminase (previously known as glutamate-pyruvate transaminase), thereby minimizing back-conversion of pyruvate to lactate. The NADH formed is measured spectrophotometrically and stoichiometrically related to D- or L-lactate concentration (Boehringer Mannheim, 1986).

Acetic acid measurement involves conversion of acetate to acetyl-CoA by acetyl-CoA synthetase with the consumption of ATP (Boehringer Mannheim, 1986). Acetyl-CoA reacts with oxaloacetate and water in the presence of citrate synthetase to form citrate and CoA. Oxaloacetate for this reaction is obtained from malate by the action of malate dehydrogenase with concomitant conversion of NADH from NAD^+. NADH is spectrophotometrically measured and correlated to acetic acid concentration.

Citrate is also an important precursor for the flavor compounds in cheeses made using mesophilic starter cultures. NSLAB metabolize citrate to acetoin, acetate, butanediol, and diacetyl. The standard methods for quantification of citrate are ISO (2006b) and AOAC (2005; methods 976.15 and 920.126). It can also be quantified enzymatically (Boehringer Mannheim, 1986) or chemically (Marier and Boulet, 1958). In the enzymatic method, citrate is converted to oxaloacetate and acetate by the enzyme citrate lyase. Oxaloacetate may be decarboxylated to pyruvate. In the presence of malate dehydrogenase and L-lactate dehydrogenase, oxaloacetate and pyruvate are converted to L-malate and L-lactate, respectively. During this process NADH is oxidized to NAD^+, which is measured spectrophotometrically at 340 nm (Boehringer Mannheim, 1986). In the chemical method, cheese is first dispersed into NaOH, followed by precipitation of proteins using trichloroacetic acid (TCA). The reaction mixture is then filtered and reacted with pyridine and acetic anhydride. The yellow color formed is measured at 428 nm.

Diacetyl and acetoin can be quantified by a colorimetric method. It involves extraction of diacetyl by steam distillation. Diacetyl is collected in the first 10 ml of the distillate and acetoin in the second 10 ml. To quantify diacetyl, the distillate is treated with hydroxylamine to form dimethylgloxime (Walsh and Cogan, 1974a,b). On reaction with $FeSO_4$ in alkaline conditions, dimethylgloxime is converted to ammonoferrous dimethyl glyoximate complex (pink colored), which is measured colorimetrically at 525 nm. Acetoin collected in the second 10 ml of the distillate is reacted with 2-naphthol and creatine to form a red complex that can be measured spectrophotometrically at 525 nm. The presence of acetolactate, produced by certain starter cultures, will cause errors in the determination of diacetyl and acetoin as it decomposes to diacetyl and/or acetoin during steam distillation (Fox *et al.*, 2004a). In this case, to determine diacetyl, the sample must be brought down to a pH of 0.5 prior to steam

distillation. Under such conditions, all of the acetolactate is converted to acetoin. Acetolactate can be quantified with a modified distillation method (Mohr *et al.*, 1997). The pH of the sample is first adjusted to 3.5 and $CuSO_4$ is added prior to distillation. This converts all acetolactate to diacetyl. The total diacetyl and the actual free diacetyl are determined as described above and subtracted to obtain the acetolactate content.

Many of the products of lactose metabolism can also be quantified by other methods. Zeppa *et al.* (2001) described a HPLC (high-performance liquid chromatography) method to determine sugars (lactose, glucose, and galactose), nonvolatile acids (citric, orotic, lactic, etc.), free fatty acids (FFAs) (formic, acetic, propionic, butyric, etc.), diacetyl, and acetoin. Dinkci *et al.* (2007) quantified lactic, acetic, citric, propionic, formic, pyruvic, uric, and orotic acid using a simple sample treatment and reverse-phase HPLC. An isocratic HPLC method was followed with UV and refractive index detection. Other HPLC methods for the simultaneous detection of sugars, organic acids, and short-chain fatty acids have also been published by Lues *et al.* (1998), Mullin and Emmons (1997), and Bouzas *et al.* (1991). Propionic acid and acetic acids, which are involved in propionic acid fermentation in Swiss cheese, can be extracted with dilute H_2SO_4 and analyzed by HPLC. Lues *et al.* (1998) compared three extraction methods and found that extraction with 0.009 N H_2SO_4 provided the best recoveries for 11 of the 16 compounds that were analyzed by ion-exchange HPLC. Sample acidification followed by steam distillation yields cleaner samples for chromatographic analysis. However, this method requires running several standards as each acid may be distilled to a different extent (Fox *et al.*, 2004a). Izco *et al.* (2002) reported a rapid and simultaneous capillary electrophoretic method for the simultaneous determination of organic acids, free amino acids, and lactose in cheese. Eleven metabolically important organic acids (oxalic, formic, citric, succinic, orotic, uric, acetic, pyruvic, propionic, lactic, and butyric) were determined with detection limits as low as 2 μM.

Gas chromatographic methods have also been developed to analyze some of the products of lactose metabolism. Harvey *et al.* (1981) analyzed glucose, galactose, lactose, lactate, and succinate by GC. Sugars and acids were modified to trimethylsilyl derivatives and methyl esters, respectively. In another study, several cheese compounds including diacetyl, acetoin, acetic acid, and 2,3-butanediol were identified and quantified by Fernández-Garcia (1996). 2,3-Butanediol can also be determined by extraction with methylene chloride, followed by separation of the residue and drying with anhydrous Na_2SO_4 (Fox *et al.*, 2004a). On partitioning the extract with water, 2,3-butanediol moves to the aqueous phase, which is then clarified with a mixture of $BaCl_2$, NaOH, and ZnSO4 and analyzed by GC to measure butanediol. The determination of acetolactate by GC involves reducing the pH of the sample to 3.1 and converting acetolactate

to diacetyl using $FeCl_3$ (Richelieu *et al.*, 1997). The pH is then adjusted to 7.0 followed by headspace capillary GC analysis to measure diacetyl.

B. Assessment of lipolysis

Lipolysis is considered to be an important biochemical event during cheese ripening and the current knowledge have been discussed in detail (Collins *et al.*, 2003, 2004; McSweeney and Sousa, 2000). The formation of short-chain FFAs by the lipolysis of milk fat by lipases is a desirable reaction in many cheese types (e.g., mold-ripened cheeses). The catabolism of FFAs, which is a secondary event in the ripening process, leads to the formation of volatile flavor compounds such as lactones, thioesters, ethyl esters, alkanols, and hydroxyl fatty acids. The contributions of lipolysis to the flavor of bacterially ripened cheeses are limited.

Colorimetric and chromatographic methods are available for the assessment of lipolysis. Several of these techniques, including sample preparation procedures, were reviewed recently by Collins *et al.* (2004). There are two colorimetric methods available for monitoring the release of FFAs: the copper soaps method (IDF, 1991) and the acid degree value (ADV) method. In the copper soaps method, developed by Koops and Klomp (1977) and Shipe *et al.* (1980) and modified by Bynum *et al.* (1984), the sample is mixed with HCl and copper soaps reagent (100 ml 1 M $Cu(NO_3)_2 \cdot 2.5H_2O$) + 50 ml triethanolamine diluted to 1 l with saturated NaCl and pH adjusted to 8.3 with 1 M NaOH). The mixture is incubated for 10 min at 60 °C, cooled and mixed. The FFAs react with $Cu(NO_3)_2$ and form copper soaps, which are partitioned with a mixture of chloroform, heptane, and methanol (49:49:2, v/v/v) and centrifugation. The solvent layer is mixed with a solution of diethyldithiocarbamate in butan-1-ol forming a copper-color complex, which is measured at 440 nm. Palmitic acid is used to develop the standard curve. This method does not efficiently partition short-chain fatty acids (<C10), which play a very important role in cheese flavor (Fox *et al.*, 2004a). This was confirmed by Ikins *et al.* (1988), who reported a good correlation for long-chain fatty acids and poor correlation for short-chain fatty acids between the copper soap method and GC.

The ADV or the Bureau of Dairy Industry (BDI) method involves extraction of FFAs using detergents, ion-exchange reactions, heat, and centrifugation. A known aliquot of the fat layer is dissolved in solvent and titrated against alcoholic KOH (~0.02 M) using methanolic phenolphthalein as the indicator (Deeth and Fitzgerald, 1976). Salji and Kroger (1981) used the BDI method with slight modifications to analyze Cheddar cheese. The sample (10 g) was taken in a butyrometer and mixed with 20 ml of BDI reagent (30 g Triton X-100 and 70 g sodium tetraphosphate, made up to 1 l with water). The mixture was incubated at 100 °C for 20 min and centrifuged to separate fat. Aqueous methanol was added to

the butyrometer to bring up the fat. The aliquot of the fat was collected and titrated with alcoholic KOH. Both the copper soaps method and ADV methods are tedious and have drawbacks. But with training and careful analysis, they may be reliable and reproducible.

The most common analytical technique for the analysis of FFAs and their breakdown products has been chromatography. HPLC has been used for the analysis of FFAs (Christie, 1997; Lues *et al.*, 1998; Zeppa *et al.*, 2001). Analysis of short-chain fatty acids (C2–C4) may be relatively simple (Zeppa *et al.*, 2001). However, the analysis of long-chain fatty acids (>C6) may require derivatization. They are extracted using solvents, converted to bromophenacyl esters, and analyzed by reverse-phase HPLC. GC (with sample preparation and derivatization) has been the method of choice for analysis of fatty acids. An ideal but difficult procedure is to extract FFAs from the aqueous phase and organic phase and combine them (IDF, 1991). The challenge is to overcome the effects of partitioning and extraction of compounds that interfere with the analysis. ISO and IDF have detailed some of the extraction methods for lipids and liposoluble compounds in milk products (ISO, 2001b). Several other methods, which are mainly different in the extraction and derivatization steps, were reviewed by Collins *et al.* (2004).

The standard jointly published by ISO and IDF (ISO, 2002a) described three categories of sample preparation and gas chromatographic analysis for the determination of fatty acids. The first category involves direct analysis (without derivatization) of the FFAs. The method of Nieuwenhof and Hup (1971) involved separation of the FFAs using an alkaline silica gel column, followed by elution, concentration, and quantification by GC. This method was later shown to cause hydrolysis of fat due to the use of silicic acid column. Another direct method used a precolumn to remove lactic acid and used a silicic acid–KOH column (Woo *et al.*, 1984). The FFAs were then separated on a glass column packed with diethylene glycol succinate (DEGS-PS) with formic acid as the mobile phase. The second category of methods involves the use of solvent extraction and methylation (Fontecha *et al.*, 1990; McNeill and Connolly, 1989). The lipid fraction is extracted using a solvent and the FFAs are converted to methyl esters to impart volatility prior to chromatography. Another commonly used direct analysis method for underivatized FFA was described by de Jong and Badings (1990). The FFAs were extracted from cheese paste containing anhydrous sodium sulfate and H_2SO_4, using solvents (ether/heptane), isolated in an aminopropyl ion-exchange column, and analyzed by GC.

The third category is the basis of many commonly used methods. In general, the steps in this category are solvent extraction to remove the lipid fraction, saponification, methylation to fatty acid esters, separation using chromatographic columns and GC analysis. Martin-Hernández

et al. (1988) and later Poveda *et al.* (1999) extracted the lipids from cheese paste using diethyl ether and saponified the FFAs using tetramethylammonium hydroxide (TMAH) to TMA-soaps. The TMA-soaps of FFA were neutralized (to pH 9.0) and analyzed by GC. The derivatization of FFAs to fatty acid methyl esters (FAME) prior to separation and GC analysis is also a very common method (Ha and Lindsay, 1990; Pavia *et al.*, 2000). The standard procedure for the preparation of FAME is available from ISO (2002b). Perotti *et al.* (2005) separated ethyl esters of FFA by chromatography. The authors extracted the FFAs in hexane and converted them to sodium salts using NaOH. The salts were then dried, esterified with ethanol–H_2SO_4, and partitioned with *n*-hexane. The upper phase containing the ethyl esters of the FFAs was separated by chromatography.

Normally, odd-numbered fatty acids are used as internal standards. While the use of internal standards ensures the correctness of the extraction procedure, it does not guarantee the completeness of extraction for different fatty acids. Due to this reason, a comparison between the methods is essential to truly determine the efficacy of extraction. Chavarri *et al.* (1997) compared two sample preparation procedures. The first method was the direct method developed by de Jong and Badings (1990), described above. The second method involved saponification with TMAH as described by Martin-Hernández *et al.* (1988) and the formation of methyl esters in the injector prior to analysis. The authors found that separation of the FFAs from the triglycerides prior to derivatization improved the analysis. Another comparative study by Ardö and Polychroniadou (1999) reported that the saponification method described above (Martin-Hernández *et al.*, 1988) was found suitable for both low and high FFA levels in cheese.

GC combined with mass spectroscopic (MS) detection provides very accurate identification and quantification of FFAs. Pinho *et al.* (2003) monitored changes in the FFA content during the ripening of ewe cheese. Sampling was done by headspace solid-phase microextraction (SPME). An excellent correlation was observed between the initial concentration of the sample and the amount absorbed on the SPME fiber. SPME sampling was done at 65 °C with a fiber coated with 85-μm polyacrylate film. After equilibration at 65 °C for 40 min, the fiber was exposed to the sample headspace for 20 min and inserted into the GC port. Despite its accuracy, the GC–MS method is not widely used, presumably because of its cost and complexity.

C. Assessment of proteolysis

Proteolysis is the most important of the three primary events occurring during cheese ripening. Due to the complexity of proteolysis, including the catabolism of amino acids and their contribution to cheese flavor, this topic has been the focus of several studies. A comprehensive review of the

available literature and the current knowledge on proteolysis and amino acid breakdown was published recently by Upadhyay *et al.* (2004). In addition, several other reports on this topic are worth mentioning (Curtin and McSweeney, 2004; Fox, 1989; Fox *et al.*, 2004a; McSweeney and Sousa, 2000; Sousa *et al.*, 2001; Yvon and Rijnen, 2001). A simple schematic diagram of proteolysis and the proteolytic agents involved is shown in Fig. 5.1. Initial proteolysis is due to the residual coagulant activity and milk proteinases such as plasmin, which hydrolyze casein to large- and intermediate-sized peptides. The proteinases from primary and secondary starters metabolize the large peptides to small peptides. The LAB and NSLAB supply peptidases that produce free amino acids by catabolizing small peptides. Amino acids are further broken down to several flavor compounds (Curtin and McSweeney, 2004).

The assessment of proteolysis is essential for analyzing the extent of ripening. The techniques available for the assessment of proteolysis have been reviewed by McSweeney and Fox (1993, 1997) and Upadhyay *et al.* (2004). A schematic diagram of some of the methods used to assess proteolysis published by Sousa *et al.* (2001) is shown in Fig. 5.2 with some modifications. A comparison of the techniques available for free amino acid analyses was compared by Lemieux *et al.* (1990) and Bütikofer and Ardö (1999). According to McSweeney and Fox (1993, 1997), these techniques can be grouped into two: nonspecific and specific.

1. Nonspecific methods for assessment of proteolysis

The nonspecific methods provide information about the extent of proteolysis and activity of proteolytic agents. Nonspecific methods are relatively simple and straightforward and, with the right sample preparation or fractionation, provide a good index of proteolysis. As the proteolysis progresses during ripening, the amount of soluble nitrogen (SN) increases. These methods measure the amount of nitrogenous compounds by extraction, precipitation, or liberation of the reactive groups. Several extraction and fractionation techniques have been developed in the past to determine cheese nitrogen (N). A review of the several extraction methods available for studying proteolytic products such as peptides

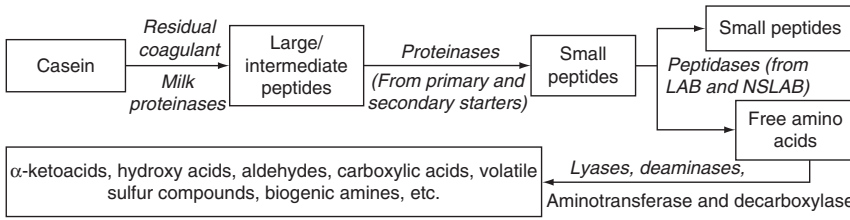

FIGURE 5.1 Overview of proteolysis during cheese ripening.

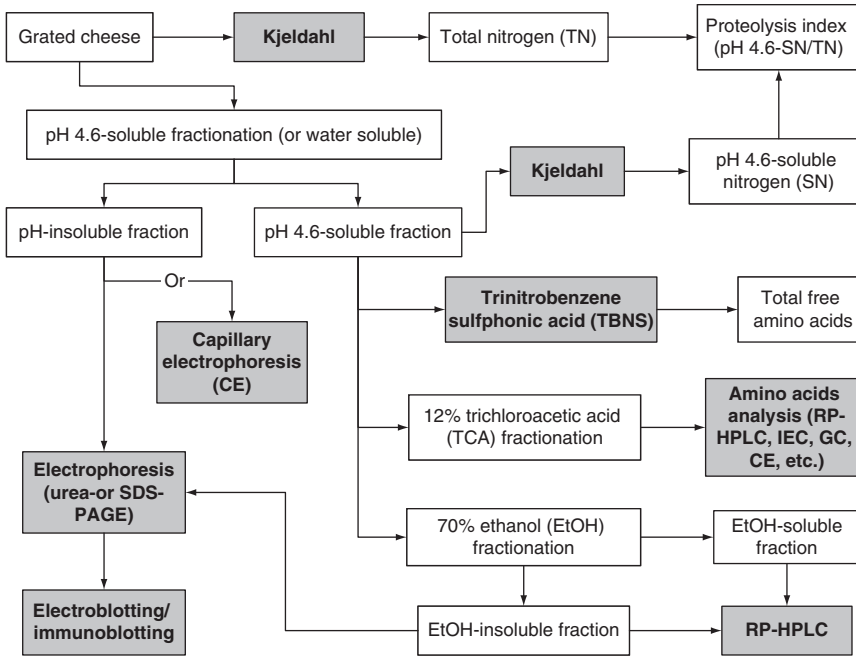

FIGURE 5.2 Analysis scheme for assessment of proteolysis during cheese ripening, modified from Sousa *et al.* (2001). Analytical techniques are highlighted in bold with shading.

and amino acids in cheese can be found in McSweeney and Fox (1993, 1997) and Fox *et al.* (1995). A summary of the various methods for the analysis of cheese N is presented in Table 5.3. The extraction or fractionation method depends on the target analyte and may be done based on selective precipitation with reagents or a separation based on molecular size (filtration).

The analysis of water-soluble extracts (WSE) is one of the most common methods to isolate, characterize, and quantify proteolytic products such as peptides and amino acids. Several procedures with water as the primary extractant have been developed and reviewed (Christensen *et al.*, 1991; McSweeney and Fox, 1993, 1997). A method used by several authors (González de Llano *et al.*, 1991; Kuchroo and Fox, 1982a; Singh *et al.*, 1995; Wilkinson *et al.*, 1992) involves homogenization of cheese in twice the amount of water in a stomacher (20 °C for 10 min). The slurry is incubated (40 °C for 1 h), centrifuged, and filtered through glass wool and Whatman no. 113 filter paper. Analysis of water-soluble nitrogen (WSN) is suitable for cheese varieties in which the pH does not change significantly during

TABLE 5.3 Some extraction and fractionation methods for the analysis of cheese nitrogen

Method	Principle/application	References
Hydraulic pressure/ centrifugation	Separates aqueous phase without altering the ionic composition. Principally applied in studies on calcium and phosphate in cheese, buffering capacity, lysis of starter cultures	Wilkinson *et al.* (1992), Lucey *et al.* (1993), and Guo and Kindstedt (1995)
Water-soluble extracts (WSE)	Commonly used to extract short peptides and amino acids. Suitable for bacterially ripened cheeses	Rank *et al.* (1985), Aston and Creamer (1986), Kuchroo and Fox (1982a,b, 1983a,b), Farkye and Fox (1991), and O'Sullivan and Fox (1990)
Extraction at pH 4.6	Suitable for young bacterially ripened cheese varieties	Kuchroo and Fox (1982a), Vakaleris and Price (1959), Vakaleris *et al.* (1960), O'Keeffe *et al.* (1978), and Reville and Fox (1978)
$CaCl_2$	Extraction of a portion of whey proteins, peptides, and amino acids	Venema *et al.* (1987) and Kuchroo and Fox (1982a)
NaCl	Suitable for extraction of caseins and peptides from young cheeses. Not as effect as water	Reville and Fox (1978), Rank *et al.* (1985), Fox (1989), and Kuchroo and Fox (1982a)
Chloroform/methanol	Extraction of bitter and astringent peptides	Harwalkar and Elliott (1971), Lemieux *et al.* (1990), Puchades *et al.* (1990), Visser *et al.* (1983), and Rank *et al.* (1985)
Trichloroacetic acid (TCA)	Precipitates water-soluble N. Ratio of 12% TCA soluble N:total N is a better index of maturity than water-soluble N:total N. TCA must remove prior to further analysis	Kuchroo and Fox (1982a,b), O'Sullivan and Fox (1990), O'Keeffe *et al.* (1978), Reville and Fox (1978), Venema *et al.* (1987), and Fox (1989)
Ethanol	Precipitates proteins and peptides. Concentration influences fractionation (70% most common). Preferred to TCA due to ease of removal of ethanol	Edwards and Kosikowski (1983), Aston and Creamer (1986), Kuchroo and Fox (1982a,b, 1983a), and Reville and Fox (1978),

(*continued*)

TABLE 5.3 (*continued*)

Method	Principle/application	References
Phosphotungstic acid (PTA)	Discriminating protein precipitant. Precipitates dibasic amino acids and peptides >600 Da. PTA soluble N is used as n index of free amino acids	Milesi *et al.* (2007), Wilkinson *et al.* (1992), Bütikofer *et al.* (1993), Bican and Spahni (1991), and González de Llano *et al.* (1987, 1991)
Sulfosalicylic acid	Used to prepare samples from amino acid analysis	Fox (1989), Kuchroo and Fox (1982a), and Ramos *et al.* (1987)
Picric acid	Suitable for extraction of amino acids and small peptides. Interferes with Kjeldahl or spectrophotometric methods	Fox (1989), Salji and Kroger (1981), and Reville and Fox (1978)
$Ba(OH)_2/ZnSO_4$	Extracts free amino acids. Not widely used extractant for N from cheese	Hickey *et al.* (1983)
Ethylenediaminetetraacetic acid (EDTA)	Used for fractionation of water-soluble extracts. Precipitates approximately 30% of water-soluble N	Kuchroo and Fox (1982b)
Dialysis and ultrafiltration	Fractionation based on molecular size. Used for fractionation of WSE for further analysis. Potential limitations include rejection of hydrophobic peptides by UF membranes and aggregation of small peptides	Kuchroo and Fox (1982b, 1983b), Visser *et al.* (1983), O'Sullivan and Fox (1990), and Fox (1989)
Trifluoroacetic acid/formic acid	Fractionation of cheese N for chromatographic and electrophoretic analyses	Bican and Spahni (1991)

the ripening process (e.g., bacterially ripened cheeses such as Cheddar and Swiss for which the pH is around 5.2). Quantification of pH 4.6-SN is another measure for the extent of proteolysis. However, like WSE, these methods are not suitable for mold and bacterial surface-ripened cheeses, which have higher pH (McSweeney and Fox, 1993, 1997). This results in a relatively (compared to water) lower extraction of N (Kuchroo and Fox, 1982a). Some applications of this extraction method to cheese analysis include Fox (1989), Kuchroo and Fox (1982a,b), O'Keeffe *et al.* (1978), Reville and Fox (1978), Vakaleris *et al.* (1960), and Vakaleris and Price (1959).

Solutions containing salts, especially $CaCl_2$ and NaCl (separately and in mixtures), may be used to fractionate or subfractionate WSE. Extraction with $CaCl_2$ with/without NaCl involves the homogenization of cheese, pH adjustment, and centrifugation (Venema *et al.*, 1987). $CaCl_2$ extracts only about 40% of the WSN (Kuchroo and Fox, 1982a) and contains whey proteins, peptides, and amino acids (Christensen *et al.*, 1991). A NaCl solution (5%) was reported to extract greater than 90% of the N in Cheddar cheese (Reville and Fox, 1978). The discrimination of the NaCl solution is low (Bican and Spahni, 1991; Rank *et al.*, 1985) but an improvement was observed with the addition of $CaCl_2$ (Fox, 1989). A comparison chart showing the formation of SN in blue and Cheddar varieties during the ripening process, studied using different extractants, was presented by McSweeney and Fox (1997).

TCA (CCl_3COOH), a protein precipitant, has been used at concentrations ranging from 2% to 12%, with 12% TCA being more common (Addeo *et al.*, 1994; Bican and Spahni, 1991; Bütikofer *et al.*, 1993; Folkerstma and Fox, 1992; Milesi *et al.*, 2007; O'Sullivan and Fox, 1990). Further analyses of these extracts require the removal of TCA, which can be problematic and may require tedious procedures including chromatography. The use of 70% ethanol (Fox, 1989) or trifluoroacetic acid (CCF_3COOH; Bican and Spahni, 1991) has been suggested, due to the ease of their removal by evaporation. Phosphotungstic acid (PTA) with H_2SO_4 is another precipitant that has been used to extract and study soluble N. It extracts only free amino acids and peptides smaller than 600 Da, at a commonly used concentration of 5% (Bican and Spahni, 1991; Bütikofer *et al.*, 1993; González de Llano *et al.*, 1991; Milesi *et al.*, 2007; Wilkinson *et al.*, 1992). A comparison of TCA- and PTA-SN in blue and Cheddar cheese with other extractants was shown by McSweeney and Fox (1997).

Fractionation with organic solvents is mainly done to remove proteins, large peptides, and non-proteinaceous material such as fat. In a method developed by Harwalkar and Elliott (1971) and adopted by Lemieux *et al.* (1990), Puchades *et al.* (1990), and Visser *et al.* (1983), freeze-dried samples of cheese were extracted using methanol (to precipitate large peptides and proteins), chloroform (to remove fat), and water. The final extract

contained bitter and astringent peptides. Precipitation with ethanol has also been studied and widely used with a 70% concentration being more common (Abdel Baky *et al.*, 1987; Christensen *et al.*, 1991; Fox *et al.*, 1995; McSweeney and Fox, 1993).

Ultrafiltration or dialysis is an example of fractionation of cheese based on molecular size and has been adopted by several authors (Kuchroo and Fox, 1982b, 1983b; Singh *et al.*, 1997; Visser *et al.*, 1983). A fractionation scheme for cheese nitrogen based on filtration can be found in several publications (Fox, 1989; Kuchroo and Fox, 1983b; McSweeney and Fox, 1993, 1997). Recently, Combes *et al.* (2002) used gel filtration and reverse-phase fast protein liquid chromatography to isolate low-molecular-weight peptides from Emmentaler cheese. Various other fractionation schemes have been proposed and tested to fractionate heterogeneous samples and isolate compounds or group of compounds of interest (Aston and Creamer, 1986; Fox, 1989; Kuchroo and Fox, 1983b; O'Sullivan and Fox, 1990).

Methods based on the liberation of reactive compounds or groups are more direct methods to estimate proteolysis and may be relatively rapid and simple. These include several colorimetric and titrimetric methods. A review of the application of these methods in cheese analysis was presented by Wallace and Fox (1998). Monitoring ammonia, which is formed during cheese ripening by deamination of amino acids, is a measure of proteolysis in certain types of cheeses. This reaction is accompanied by an increase in pH (in mold- and smear-ripened cheeses). Hence, changes in pH have been used as an indirect measure of ammonia production (Furtado and Chandan, 1985). The spectrophotometric method relies on the amount of tyrosine and tryptophan to measure proteolysis. The two amino acids are quantified in fractionated cheese at 280 nm (Vakaleris and Price, 1959). They can also be quantified by their reaction with Folin–Ciocalteau reagent (Singh and Ganguli, 1972) or by titration (Fernandez-Salguero *et al.*, 1989).

Formol titration is a method that estimates amino groups by titration with NaOH and a phenolphthalein indicator (Vakaleris *et al.*, 1960). Addition of formaldehyde to the neutralized mixture reduces the pH by making the amino groups less basic. The amount of NaOH required to retitrate the mixture has been used as an indicator of proteolysis. Another titrimetric method relies on the increase in buffering capacity of the cheese during ripening and has been applied to study proteolysis in Swiss cheese (Lucey *et al.*, 1993).

The dye-binding properties of extracts from cheese have been investigated as an index of ripening (Basch *et al.*, 1989; Kroger and Weaver, 1979). These methods are based on the principle that at a pH below the isoelectric point, proteins have net positive charge and bind with anionic dyes such as amido black and acid orange 12. Protein–dye complexes are removed by filtration/centrifugation and the reduction in color is proportional to the protein concentration.

Proteolysis in cheese has also been measured based on fluorimetric and colorimetric methods. The color or fluorescence formed on binding of free amino groups to a chromophore or a fluorophore is monitored. The chemical reaction between amino groups and various flurophores and chromophores has been detailed previously (McSweeney and Fox, 1997). 2,4,6-Trinitrobenzene sulfonic acid (TNBS) is a chromophore that binds to amino acids, peptides, and proteins and absorbs at 420 nm. Several researchers have applied this technique to monitor proteolysis (Ardö and Meisel, 1991; Barlow *et al.*, 1986; Bouton and Grappin, 1994; Clegg *et al.*, 1982; Kuchroo *et al.*, 1983). The TNBS method correlated well with several other methods of protein or nitrogen determination. Although this method is simple, the activity of TNBS changes over time and, in dry form, TNBS is explosive. Ninhydrin is another chromophore that reacts with amino groups and absorbs at 570 nm (Ardö and Meisel, 1991). Additionally, it also reacts with ammonia resulting in overestimation (Clegg *et al.*, 1982). But ninhydrin is more sensitive than TNBS and is widely used to quantify amino groups in various samples, including chromatographic eluates (Cliffe *et al.*, 1989). Folkerstma and Fox (1992) found Cd-ninhydrin to be five times as sensitive as TNBS for the measurement of amino acids. Modified versions of this assay using Li-ninhydrin (Friedman *et al.*, 1984; Pearce *et al.*, 1988), hydrindatin (Doi *et al.*, 1981; Moore and Stein, 1954), and Cd-ninhydrin (Doi *et al.*, 1981; Folkerstma and Fox, 1992) also exist. A comprehensive review of the applications of ninhydrin reactions for the analysis of amino acids, peptides, and proteins in biological samples was published recently by Friedman (2004).

Fluorimetric methods for the determination of amino acids are generally more sensitive than colorimetric methods. Fluorescamine (4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione) and *o*-phthaldialdehyde (OPA) substances are used for protein analysis. Fluorescamine reacts with amino groups to form fluorophores that excite at 390 nm and emit at 475 nm (Weigele *et al.*, 1972). Applications of fluorescamine include monitoring the hydrolysis of κ-casein (Beeby, 1980; Pearce, 1979) and quantification of proteins, peptides, amino acids in extracts (Creamer *et al.*, 1985). OPA produces fluorescence on reaction with 2-mercaptoethanol and primary amines, with strong absorption at 340 nm. Lemieux *et al.* (1990) claimed that this method was more accurate, convenient, and simple for estimating free amino acids than the TNBS, ninhydrin, or fluorescamine methods.

Many enzymatic assays have also been developed for the analysis of proteolytic products. Total amino acids in Cheddar cheese were determined by Puchades *et al.* (1990) using the L-amino acid oxidase enzyme. Glutamic acid has been quantified by flow injection analysis using glutamate dehydrogenase (Puchades *et al.*, 1989) and using the Boehringer-Mannheim kit (McSweeney *et al.*, 1993).

2. Specific methods for assessment of proteolysis

Specific methods are used to isolate and identify specific peptides formed during ripening. In general, the widespread application of specific methods to cheese analysis is more recent than nonspecific methods. However, these methods have provided valuable information and played a significant role in furthering the understanding on cheese ripening. Electrophoresis and chromatography are the most widely used methods in this category.

a. Electrophoresis Electrophoresis is a separation technique used for the separation of proteins and peptides based on their migration patterns in an electric field and/or based on their molecular size. Several types of electrophoresis, including polyacrylamide gel electrophoresis (PAGE), urea-PAGE, sodium dodecyl sulfate-PAGE (SDS-PAGE), two-dimensional (2-D) electrophoresis, and capillary electrophoresis (CE) have been applied to cheese analysis. Electrophoretic methods for assessment of proteolysis have been reviewed and compared by Creamer (1991) and Shalabi and Fox (1987). The majority of the applications have been on monitoring primary proteolytic products from casein. Although a few applications of electrophoresis with paper (Lindqvist *et al.*, 1953), starch (van den Berg and de Koning, 1990), and cellulose acetate (Deshmukh *et al.*, 1989) for cheese protein analyses exist, recent applications have used polyacrylamide gels.

The preparation of samples for electrophoresis involves dispersing the cheese sample into a buffer. Most of the recent applications use a discontinuous buffer system, which enhances the sharpness of the bands by creating an initial ion gradient. For applications in studying cheese protein, the buffer usually contains a reducing agent such as 2-mercaptoethanol or dithiothreitol that breaks the tertiary and quaternary structure by reducing the disulfide bonds. The solution is then centrifuged to separate fat. The density of the final sample is increased by adding glycerol or sucrose prior to loading the gels (Creamer, 1991). Glycerol also helps maintain sample stability during storage. Polyacrylamide gels are available at various concentrations (generally between 5% and 25%) and many times gels with concentration gradients (e.g., 9–19% by Gaiaschi *et al.*, 2001) may be used. The staining options available for electrophoresis were compared by Shalabi and Fox (1987). A direct staining procedure developed by Blakesley and Boezi (1977) with Coomassie Blue G-250 or Amido Black 10B in the presence of TCA was reported to be suitable for primary degradation products and silver staining technique for low-molecular-mass peptides. Silver staining is almost 100-fold more sensitive than Coomassie Blue but has a high background, contains noise from other compounds, and is expensive. Recently, Pappa *et al.* (2008) used

both Coomassie Blue and silver staining to analyze proteins and peptides in Teleme cheese by both 1-D and 2-D electrophoresis.

The use of denaturing agents such as urea or SDS is also common. SDS denatures the secondary and part of the tertiary (nondisulfide links) structures of proteins and applies a negative charge to the protein. Hence the movement of proteins/peptides in this case is due to molecular size. The urea-PAGE procedure described by Andrews (1983), who used an alkaline Tris buffer containing urea, was recommended by Shalabi and Fox (1987). Pino *et al.* (2009) and Rehman *et al.* (2003) monitored the proteolysis in goat's milk cheese and the effect of adding dry milk protein concentrate to pizza cheese, respectively, using urea-PAGE. Several other authors have used urea-PAGE, with some of the applications based on the Andrews (1983) method, to monitor proteolysis (Gardiner *et al.*, 1998; Guo and Kindstedt, 1995; Guo *et al.*, 1997; Mayer *et al.*, 1998; O'Malley *et al.*, 2000). The most commonly used standard for comparison purposes is sodium caseinate. Example applications of SDS-PAGE for cheese analysis include Basch *et al.* (1989), Gaiaschi *et al.* (2001), Guo *et al.* (1997), and Jin and Park (1995).

Isoelectric focusing (IF) resolves proteins and peptides based on the differences in their isoelectric points (pI). It has been mainly used in studying casein breakdown (Moio *et al.*, 1988). Amigo *et al.* (1992) did not find any significant difference between PAGE and IF in analyzing Serra da Estrela cheese. Other reports include the detection of casein proteolysis in several cheese varieties (Addeo *et al.*, 1995) and the analysis of milk sources in cheese based on the casein fractions (Mayer *et al.*, 1997). IF has also been combined with SDS-PAGE in a 2-D electrophoresis to assess proteolysis in Cheddar and Camembert cheeses (Chin and Rosenberg, 1998; Trieu-Cout and Gripon, 1982). 2-D electrophoresis separates protein mixtures, based on two properties. Initial separation is 1-D and based on a single property but as the proteins move they are separated in a perpendicular direction from the first dimension based on a second property. Molecules are more effectively separated in 2-D than 1-D electrophoresis. However, it is time-consuming and tough to reproduce (Creamer, 1991). A recent application of 2-D SDS-PAGE electrophoresis to study Teleme cheese was presented by Pappa *et al.* (2008).

Data from electrophoresis is normally recorded photographically. Densitometry may also be performed on the stained gel or bands may be excised/eluted for further analysis (Mayer *et al.*, 1998). The bands may also be isolated from the gels by blotting methods such as electroblotting (McSweeney *et al.*, 1994; O'Malley *et al.*, 2000) or immunoblotting (Addeo *et al.*, 1995; Moio *et al.*, 1992) for further characterization and identification. All of the electrophoretic methods, to a certain extent, provide good quality data. But due to the difficulty in quantitative analysis, very few examples are available on the quantification of protein fractions using

electrophoresis (de Jong, 1975; Deshmukh *et al.*, 1989). Furthermore, electrophoresis requires analysis of several control samples to draw reliable inferences.

CE overcomes many of the limitations of regular electrophoresis and allows for good quantitative analysis. CE separates protein fractions based on their size-to-charge ratio inside a small capillary tube filled with a buffer. The applications of CE for the analysis of food (Frazier and Papadopoulou, 2003) and dairy (Recio *et al.*, 1997) products have been reviewed. CE offers several advantages such as automatic analysis, coupling with advanced detection methods including HPLC, and continuous monitoring of UV absorbance (which in turn enables measuring small peptides). There are several types of CE available (Lindeberg, 1996; McSweeney and Fox, 1997; Zeece, 1992): free solution capillary electrophoresis (FSCE), micellar electrokinetic chromatography (SDS is used to partition peptides), capillary isotachophoresis (analyte is sandwiched between a leading and terminating electrolyte), capillary gel electrophoresis (separation based on size), and capillary electrochromatography (uses a capillary column with a coated stationary phase). Applications of this technique for monitoring cheese protein have been increasing (Miralles *et al.*, 2006; Molina *et al.*, 1998; Recio *et al.*, 1997; Zeece, 1992). CE has also been shown to be capable of quantifying free amino acids in cheese, along with organic acids and lactose (Izco *et al.*, 2002). Ten amino acids (Asp, Glu, Try, Gly, Ala, Ser, Leu, Phe, Lys, and Trp) were quantified with detection limits as low as 2 μM.

b. Chromatography Several types of chromatography have been applied to analyze cheese proteolysis. Many of the earlier applications used paper chromatography (Ardö and Gripon, 1991; Kosikowski, 1951; Kuchroo and Fox, 1982b, 1983a; O'Keeffe *et al.*, 1978), thin-layer chromatography (TLC; Bican and Spahni, 1991; Edwards and Kosikowski, 1983; Kuchroo and Fox, 1982b, 1983a,b), or column chromatography (Kuchroo and Fox, 1983a; Mojarro-Guerra *et al.*, 1991; Mulvihill and Fox, 1979). Recent methods for the analysis of peptides and amino acids have been based on ion-exchange chromatography (IEC), size exclusion chromatography (SEC), and reverse-phase HPLC (RP-HPLC).

IEC and SEC are very good techniques for fractionating large breakdown products from casein. Anion-exchange chromatography has been used to isolate casein fractions from Cheddar cheese (Creamer and Richardson, 1974), fractionate water-insoluble peptides (McSweeney *et al.*, 1994), and separate WSE from Cheddar cheese (O'Sullivan and Fox, 1990). Ion-exchange columns containing diethylaminoethyl (DEAE)-cellulose, a positively charged resin, are the most common type. Urea buffers are normally used to prepare samples, and the chromatograms are monitored at 280 nm. Peptides may be fractionated based on

their molecular weights using SEC. Sephadex gels have been the most widely used medium. Peptides in cheese extracts (in water, PTA, etc.) and ultrafiltration fractions, etc. have been separated by SEC chromatography (González de Llano *et al.*, 1987, 1991; Kuchroo and Fox, 1983b; Singh *et al.*, 1994, 1995). Monitoring is done by measuring the absorbance at 280 and 220 nm (for smaller peptides that may not contain aromatic groups), or by reaction with ninhydrin. The development of high-performance ion-exchange chromatography (HPIEC) and high-performance size exclusion chromatography (HPSEC) has simplified the analysis significantly and increased the speed and reproducibility. HPIEC columns, made of DEAE-cellulose, have been used due to their resolving power (Haasnoot *et al.*, 1989; St. Martin and Paquin, 1990). HPSEC provides better resolution of peptides and gives information about primary and secondary proteolysis. Superose-12, TSK 3000SW, and 2000SW columns have been used (Breen *et al.*, 1995; Haasnoot *et al.*, 1989; Wilkinson *et al.*, 1992). However, Haasnoot *et al.* (1989) reported that this technique may not be suitable for the separation of water- or TCA-extracts.

Amino acid analyzers have improved the analysis of free amino acids to a great extent. They offer superior sensitivity, speed, and accuracy to conventional methods. Many such systems are based on IEC. Postcolumn detection is done by ninhydrin derivatization followed by photometric measurement at 570 and 440 nm for primary and secondary amino acids, respectively. Amino acid analyzers are now common and are being manufactured by many companies (e.g., Hitachi, Beckman, PerkinElmer, HP, Pharmacia, etc.). Numerous authors have used amino acid analyzers to monitor proteolysis in several kinds of cheeses (Ardö and Gripon, 1995; Edwards and Kosikowski, 1983; Fenelon *et al.*, 2000; Gardiner *et al.*, 1998; Kaiser *et al.*, 1992; Yvon *et al.*, 1997). A comparison of amino acid analyzers and several other methods for amino acid analysis is available from Bütikofer and Ardö (1999) and Lemieux *et al.* (1990).

RP-HPLC is the most widely used technique to characterize the breakdown products of casein, especially short peptides. WSE from cheese (González de Llano *et al.*, 1991; McSweeney *et al.*, 1993), pH 4.6-soluble and -insoluble fractions (Feeney *et al.*, 2002), 10 kDa UF permeate (Singh *et al.*, 1994), ethanol extract (Feeney *et al.*, 2002), and eluates from SEC (Cliffe *et al.*, 1993) have been analyzed by RP-HPLC. The most commonly used solvent systems have been water/acetonitrile (Amantea *et al.*, 1986; Molina *et al.*, 1998), and water/methanol (Cliffe *et al.*, 1993). A C_{18} column with a stepwise acetonitrile gradient (Singh *et al.*, 1994, 1995) or C_8 column with an acetonitrile/water gradient and TFA ion-pair reagent may be used. An internal standard such as norleucine is used for quantification purposes. Applications on monitoring casein hydrolysates (Le Bars and Gripon, 1993; McSweeney and Fox, 1993; Veloso *et al.*, 2002) and the proteolysis of casein (Addeo *et al.*, 1994; Feeney *et al.*, 2002; González de

Llano *et al.*, 1991; McGoldrick and Fox, 1999; McSweeney *et al.*, 1994; Poveda *et al.*, 2003; Shakeel-Ur-Rehman *et al.*, 2000; Sousa and Malcata, 1998; Trujillo *et al.*, 2002) have been explored. Detection is usually done at UV wavelengths 200–230 nm (for carbonyls in peptides bonds or short peptides) and 280 nm (for aromatic groups or large peptides), although fluorescence detection has also been reported (González de Llano *et al.*, 1991; Verdini *et al.*, 2004). RP-HPLC combined with mass spectrometry (MS) has provided an understanding of the proteolytic pathways during cheese ripening. Several peptide fragments have been separated by RP-HPLC and subsequently identified by MS (Alli *et al.*, 1998; Gagnaire *et al.*, 2001; Gouldsworthy *et al.*, 1996). Molina *et al.* (1998) compared RP-HPLC and CE for monitoring cheese proteolysis and reported that the CE method had a shorter analysis time but the RP-HPLC method had better detection limits and precision.

Amino acid analysis by RP-HPLC is also a widely applied technique. Generally, prior to amino acid analysis, samples are treated with reagents such as sulfosalicylic acid (Verdini *et al.*, 2004), ethanol (Polychroniadou and Vlachos, 1979), picric acid (Shindo *et al.*, 1980), TCA (García-Palmer *et al.*, 1997), or $Ba(OH)_2/ZnSO_4$ (Hickey *et al.*, 1983). These reagents deprotonize amino acids and reduce interference from peptides. Detection is done using a UV or fluorescent detector. Additionally, derivatization may be required prior to HPLC analysis. OPA, dabsyl chloride, dansyl, phenylisothiocyanate (PITC), and *N*-(9-fluorenylmethoxycarbonyl) may be used for the derivatization of amino acids. García-Palmer *et al.* (1997) detected 23 free amino acids in Mahón cheese using RP-HPLC. The solvent system consisted of two eluants (sodium acetate/acetonitrile and acetonitrile/water/methanol). L-Methionine sulfonate was used as the internal standard and the amino acids were derivatized with PITC. Absorbance was monitored at 254 nm. A few other authors have quantified amino acids using RP-HPLC (Barcina *et al.*, 1995; Izco *et al.*, 2000; Pinho *et al.*, 2001; Verdini *et al.*, 2002).

Although not very common, amino acids have also been analyzed by capillary GC (Laleye *et al.*, 1987). Derivatization can be done with heptafluorobutyric anhydride (HFBA) to form *N*-heptafluorobutyryl isobutyl derivatives. The accuracy and speed are comparable to amino acid analyzers. The availability of derivatization kits (e.g., EZ:faast^{TM} Amino Acid Analysis Kit, Phenomenex Inc.) have simplified amino acid analysis by GC. Subramanian *et al.* (2009a,b) identified and quantified 20 amino acids in Cheddar cheese using the EZ:faast^{TM} kit and GC with a flame ionization detector (FID). Cheese samples were fractionated using water, chloroform, and ethanol as shown in Fig. 5.3. The total analysis time per sample, including extraction, derivatization, and GC analysis, was about 25 min. Norvaline was used as the internal standard. Figure 5.4 shows the GC-FID chromatogram of free amino acids in Cheddar cheese. This method had good reproducibility and speed.

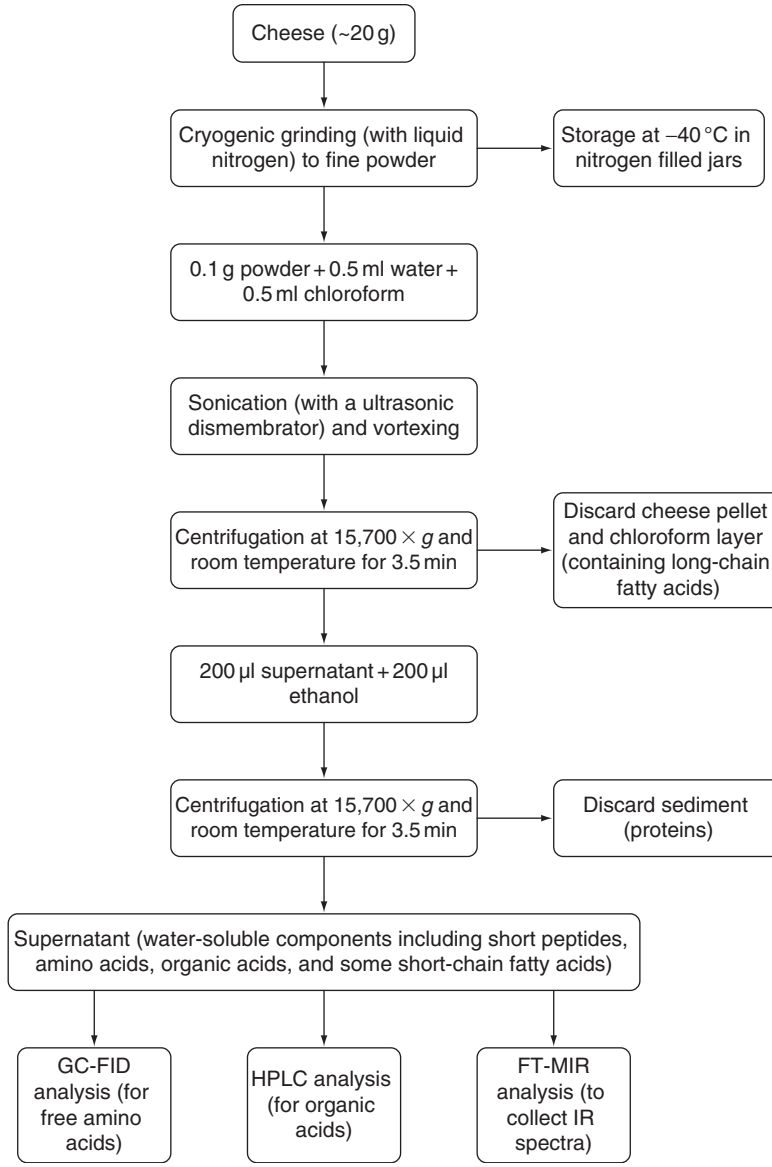

FIGURE 5.3 A fractionation scheme for extraction of water-soluble components from cheese (Subramanian *et al.*, 2009a,b).

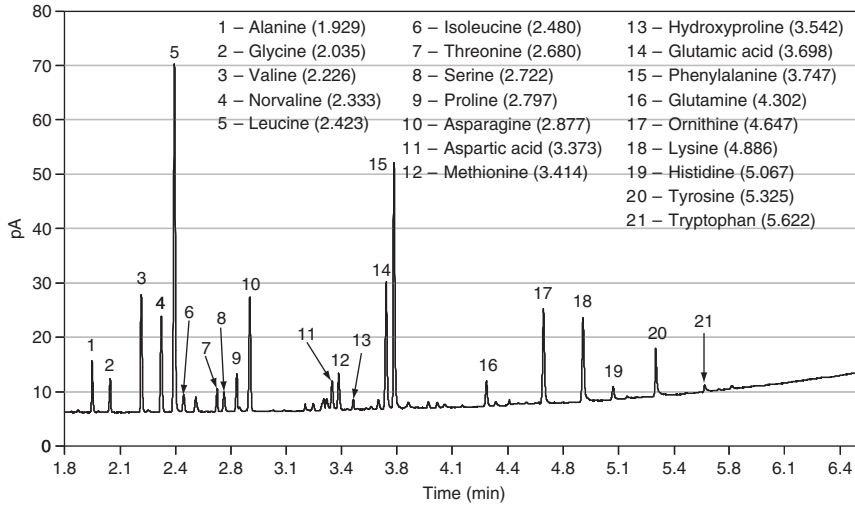

FIGURE 5.4 GC-FID chromatogram of Cheddar cheese extract showing the amino acid profile. Norvaline was used as the internal standard (IS).

D. Assessment of smaller breakdown products

Free amino acids are further catabolized into several volatile flavor compounds. However, the pathways involved are not fully known. A detailed summary of the various studies on the role of the catabolism of amino acids in cheese flavor development was published by Curtin and McSweeney (2004). Two major pathways have been suggested: (1) aminotransferase or lyase activity and (2) deamination or decarboxylation. Aminotransferase activity results in the formation of α-ketoacids and glutamic acid. The α-ketoacids are further degraded to flavor compounds such as hydroxy acids, aldehydes, and carboxylic acids. α-Ketoacids from methionine, branched-chain amino acids (leucine, isoleucine, and valine), or aromatic amino acids (phenylalanine, tyrosine, and tryptophan) serve as the precursors to volatile flavor compounds (Yvon and Rijnen, 2001). Volatile sulfur compounds are primarily formed from methionine. Methanethiol, which at low concentrations, contributes to the characteristic flavor of Cheddar cheese, is formed from the catabolism of methionine (Curtin and McSweeney, 2004; Weimer *et al.*, 1999). Furthermore, bacterial lyases also metabolize methionine to α-ketobutyrate, methanethiol, and ammonia (Tanaka *et al.*, 1985). On catabolism by aminotransferase, aromatic amino acids yield volatile flavor compounds such as benzaldehyde, phenylacetate, phenylethanol, phenyllactate, etc. Deamination reactions also result in α-ketoacids and ammonia, which add to the flavor of

cheese. However, decarboxylation of amino acids can cause flavor defects by producing biogenic amines.

Several of the smaller volatile compounds formed from the catabolism of products of primary proteolysis (e.g., amino acids) can be determined by GC. The development of capillary columns and interfacing GC with MS has noticeably increased the sensitivity of this analysis. Over 200 volatile compounds have been identified in Cheddar cheese. A list of several of these compounds can be found elsewhere (Fox *et al.*, 2004a; Singh *et al.*, 2003). The instrumental techniques available for the characterization of cheese aroma were also discussed recently (Le Quéré, 2004; Singh *et al.*, 2003).

Lactones and methyl ketones contribute to the overall flavor of cheese. Their analysis normally requires solvent extraction, distillation, and derivatization. Various extraction procedures have been developed for the analysis of lactones by GC (Wong *et al.*, 1975). Methyl ketones have been analyzed by paper chromatography (Morgan and Anderson, 1956), spectrophotometry (Godinho and Fox, 1981), and gas chromatography (Keen and Walker, 1974; Manning, 1978).

Many of the volatile compounds in cheese, including hydrocarbons, aldehydes, ketones, esters, and sulfur compounds, can be determined by GC. Development and improvements in sampling techniques such as headspace analysis (static and dynamic) and SPME have reduced the workload in the analysis of cheese volatiles by GC. Headspace analysis has been used by several authors to study volatiles in Cheddar cheese (Manning and More, 1979; Manning and Robinson, 1973; Massouras *et al.*, 2006; Price and Manning, 1983). Highly volatile compounds such as H_2S, methanethiol, and dimethyl sulfide, which are important for Cheddar flavor, were analyzed. Dynamic headspace analysis requires a larger amount of sample. The sample is purged with the carrier gas and concentrated in a trap (e.g., poly-2,6-diphenyl-*p*-phenylene oxide also known as Tenax) prior to analysis. Engels *et al.* (1997) compared volatile compounds in the water-soluble fraction of various types of ripened cheeses using dynamic headspace GC–MS analysis. A total of 53 components (including fatty acids, esters, aldehydes, alcohols, ketones, and sulfur compounds) were identified. Many other researchers have developed dynamic headspace techniques to analyze cheese (Arora *et al.*, 1995; Lawlor *et al.*, 2002; Thierry *et al.*, 1999). SPME is a relatively recent sampling technique that enables sample concentration from various sources with little or no use of solvent (Kataoka *et al.*, 2000). It is fast, relatively cost-effective, and requires a small amount of sample. Several different coatings are available for the SPME fiber: polydimethylsiloxane (PDMS), polyacrylate (PA), carboxen/polydimethylsiloxane (CAR-PDMS), and carbowax/divinylbenzene (CW-DVB). The performance of these fibers for the analysis of Cheddar cheese flavor has been evaluated and

compared (Chin *et al.*, 1996; Lecanu *et al.*, 2002). Volatile aroma compounds were identified by multivariate statistical analysis. Major cheese volatiles such as fatty acids and lactones were readily extracted by PDMS and PA but volatile sulfur compounds were not. PDMS-DVB followed by CAR-PDMS showed the highest selectivity. Getting reproducible results using SPME is a challenge, and the analysis of trace volatile compounds is difficult. Although heating can improve the recovery of trace compounds, it may also alter the sample. Few new methods such as gas chromatography–olfactometry, aroma extract dilution analysis (AEDA), and aroma extract concentration analysis (AECA) have been applied to cheese flavor analysis to identify key aroma compounds (Singh *et al.*, 2003). While these techniques have seen several developments in the past decade, they still suffer from problems such as the loss of labile compounds during distillation and long analysis times.

Biogenic amines (e.g., histamine, tyramine, tryptamine, purtricine, cadaverine, and phenylethylamine) are biologically active and have physiological importance. Early analytical procedures were based on fluorometry and TLC. These techniques are now considered laborious and error-prone, when compared to GC or HPLC. GC analysis involving derivatization to TFA (Spector *et al.*, 1963), *N*-heptafluorobutyryl isopropyl (Laleye *et al.*, 1987), and perfluoropropionyl (Staruszkiewicz and Bond, 1981) derivatives have been reported. HPLC is the widely adopted method for analyzing biogenic amines. Derivatizations using *o*-pthalicdicarboxaldehyde (Staruszkiewicz, 1977) and dansyl chloride (Antila *et al.*, 1984) have been used. HPLC methods for analyzing amines without derivatization are also available (Chang *et al.*, 1985). Some researchers have simultaneously determined amino acids and biogenic amines in cheese with dabsyl derivatization (Krause *et al.*, 1997; Pinho *et al.*, 2001).

V. NOVEL AND RAPID INSTRUMENTAL METHODS

Chemical and instrumental (e.g., chromatography and mass spectrometry) methods have provided valuable information that lead to the advancement of cheese science. However, these techniques suffer from one or more of the following problems: (1) the extensive use of solvents and gases that are expensive and hazardous, (2) high costs, (3) the requirement of specific accessories for different analytes, (4) the requirement of extensive sample preparation to obtain pure and clean samples, and (5) labor-intensive operation. These disadvantages have prompted for the evaluation and adoption of new, rapid, and simple methods such as Fourier-transform infrared (FTIR) spectroscopy. Many books are available on the basics of FTIR spectroscopy and its applications (Burns and Ciurczak, 2001; Sun, 2009). FTIR spectroscopy monitors the vibrations

exhibited by molecules under infrared light. Fourier-transform near-infrared (FT-NIR) monitors overtone and combination bands in the NIR region (10,000–4000 cm^{-1}) from O–H, C–H, S–H, and N–H stretching. FT-MIR uses lower energy (4000–400 cm^{-1}) and provides information on fundamental vibration and stretching of molecules. Although FTIR spectroscopy is almost a century old, its application to dairy received good attention only during the last 2–3 decades.

Commercially available FTIR spectrometers for rapid food analysis in the food industry are primarily based on NIR (e.g., FOSS NIRSystems, Inc., Laurel, MD). These systems are capable of determining several properties including, moisture, fat, and protein, in just a few minutes. An AOAC standard method is available for the determination of the fat, protein, lactose, and total solids content of milk by infrared spectroscopy (AOAC 972.16, 2005). Wittrup and Nørgaard (1998) developed NIR models to measure fat, nitrogen, dry matter, soluble nitrogen, amino acid nitrogen, and ammonia nitrogen in semihard Danbo cheese. Fat, nitrogen, and dry matter models showed very good correlation to traditional methods. The use of NIR spectroscopy for the measurement of cheese components such as moisture, fat, protein, etc. (Adams *et al.*, 1999; Rodriguez-Otero *et al.*, 1995), peptides in cheese (González-Martin *et al.*, 2009), and free amino acids during ripening (Skeie *et al.*, 2006) have also been reported.

The ability of MIR to monitor fundamental vibrations of several functional groups provides a new tool for researchers to look at minor compounds in cheese. Some of its early applications were focused on the analysis of macromolecules in cheese such as fat, moisture, and protein (Chen *et al.*, 1998; McQueen *et al.*, 1995). More recently, the chemical parameters of cheese (Martín-del-Campo *et al.*, 2007), composition (Rodriguez-Saona *et al.*, 2006), protein structure and interactions during ripening (Mazerolles *et al.*, 2001), and ripening of Swiss cheese (Martín-del-Campo *et al.*, 2009) were analyzed with improved techniques. Almost all attempts have been directed toward the determination of macromolecules in cheese. This is mainly because of difficulties in sampling procedures and the heterogeneous nature of cheese (McQueen *et al.*, 1995) that make analysis of minor compounds difficult.

Majority of the sampling techniques described in the literature involve the direct analysis of pieces of cheese, with the primary aim of simplifying the procedure and reducing the analysis time. Our research group has believed that in order to fully tap the potential of FTIR spectroscopy, minimize interferences from the cheese matrix, and successfully analyze minor compounds, a simple extraction procedure may be required. Several extraction methods were discussed earlier in this chapter. However, none of them were adopted for the FT-MIR analysis of cheese until Koca *et al.* (2007) analyzed the WSE of Swiss cheese to determine short-chain fatty acids. A comparison of the analysis of WSE with the direct analysis of

cheese clearly showed a significant increase in predictive capability of PLSR models. A correlation coefficient of >0.90 was reported for the prediction of acetic, propionic, and butyric acid contents and the FT-MIR spectra.

Cheese flavor quality was recently analyzed by FT-MIR for the first time using WSE (Subramanian *et al.*, 2009a). The fractionation method (shown in Fig. 5.3) involved the removal of long-chain fatty acids (using chloroform) and the precipitation of large proteins (using ethanol), which caused variations in the spectra. The extracts provided consistent spectra with well-defined peaks and very less variation between replication. The FT-MIR spectra of raw cheese and cheese WSE are shown in Fig. 5.5. The removal of long-chain fatty acids and large proteins can be confirmed from the reduction in absorption in the regions 3000–2800 and 1675–1525 cm^{-1}, respectively. Using the FT-MIR spectra, samples were classified based on their flavor quality (fermented, unclean, sour, good Cheddar, and so on). Later this method was used to predict the moisture, pH, salt, and fat content of Cheddar cheese (Subramanian *et al.*, 2009b). The coefficient of correlation between the FT-MIR spectra of the extract and reference methods were >0.92. This technique was also extended to rapidly and simultaneously predict 20 amino acids and three organic acids in Cheddar cheese (Subramanian, 2009). The regression models developed, correlating the amino acid concentrations (determined by GC-FID as explained earlier in this chapter) and organic acid concentrations (determined by RP-HPLC) to FT-MIR spectra, showed very good

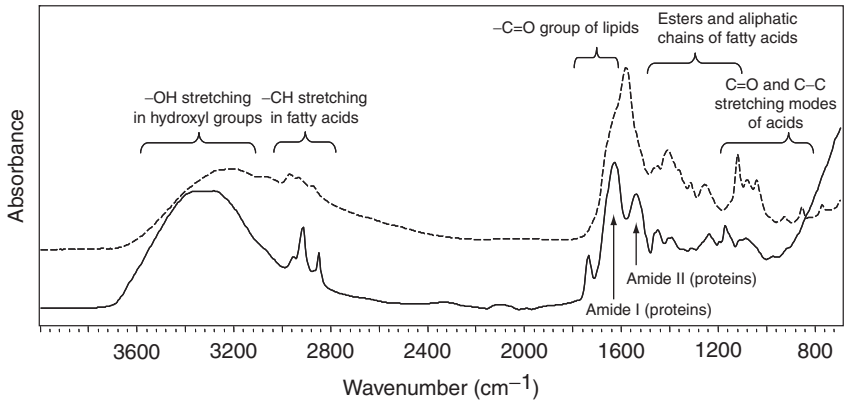

FIGURE 5.5 FT-MIR spectra of Cheddar cheese (—) and Cheddar cheese WSE (- - -). Some important functional groups and their region of absorbance are highlighted. Cheddar cheese was scanned by pressing 0.5 g of cheese on a diamond attenuated total reflectance (ATR) crystal. Extracts was scanned by drying 10 μl of the extract on a zinc selenide ATR crystal.

linear fit with the correlation coefficients >0.90 for most analytes. As an example, the regression model developed for prediction of alanine is shown in Fig. 5.6. Other authors have used this extraction method to correlate Swiss cheese descriptive sensory analysis data to FT-MIR spectra (Kocaoglu-Vurma *et al.*, 2009) and to study the effect of adjunct cultures during Swiss cheese ripening (Chen *et al.*, 2009) and observed excellent correlations.

Several new methods and instruments based on infrared spectroscopy are being developed for food applications. Advances in spectroscopic instruments and data analysis have enabled the rapid and nondestructive analysis of cheese parameters in just a few seconds (e.g., Nicolet Antaris FT-NIR by Thermo Electron Corp.). Another recent development is the miniaturization of FTIR instrumentation, which would enable onsite analysis, while the cheese is being produced. The TruDefender™ FT handheld FTIR by Ahura Scientific, Inc. (Fig. 5.7) is a portable handheld spectrometer that could be applied to food analysis. With numerous developments in FTIR spectroscopy and several potential food analysis applications still unexplored, there is great research potential in this technique that could benefit the industry and research institutions.

Several other techniques such as electronic nose (Hodgins, 1997; Schaller *et al.*, 1998) and ultrasonic methods (Benedito *et al.*, 2000; Cho *et al.*, 2001) have also been investigated. However, these methods need

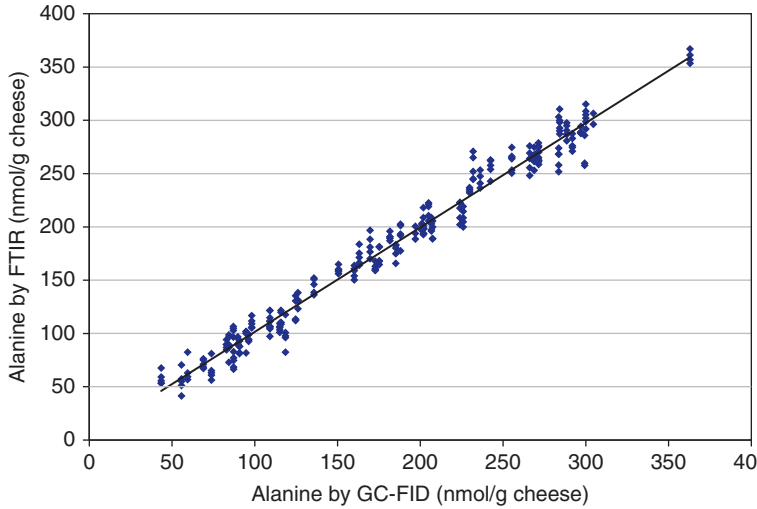

FIGURE 5.6 Partial least-squares regression model showing the correlation between alanine (nmol/g cheese) predicted by GC-FID and FTIR. The model shows a high degree of linear correlation (r-value = 0.99) and a low estimated standard error of prediction (12.70 nmol/g cheese).

FIGURE 5.7 TruDefender™ FT handheld FTIR (copyright Ahura Scientific, Inc., Wilmington, MA).

further research before they can be established as reliable research or analytical tools.

VI. CONCLUDING REMARKS

Cheese is a complex matrix of several components. Isolation of compounds of interest and the analysis of target compounds without interference from the matrix has been a challenge with analytical techniques. With the development of extraction procedures and new sampling techniques for analysis, not only has this challenge been overcome to a certain extent but also the speed, quality, accuracy, and reliability of analysis have improved tremendously. With the mechanisms behind the formation of several flavor compounds in cheese still not clearly understood, these techniques have an increasing role in the efforts to understanding cheese ripening. Often times most of the techniques provide

complementary information and what one technique may not provide may be inferred from other(s). The integration of results from several chemical and instrumental analyses in combination with sensory analysis may be required to reliably characterize cheese flavor and ripening changes.

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