12 Nutrition

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Key points

- Estimation of energy and protein requirements.
- Optimal nutrient needs for health and well-being.
- Nutrient needs during the life cycle.
- Special needs for growth, pregnancy and lactation.
- Food sources rich in nutrients.

12.1 Introduction

Nutrition may be defined as "The science of food, the nutrients and substances therein, their action, interaction, and balance in relation to health and disease, and the process by which the organisms ingest, digest, absorb, transport, utilize, and excrete food substances" (The Council on Food and Nutrition of the American Medical Association). The discovery of vitamins in the early part of the twentieth century (1906) may be viewed as a landmark in the inception of the science of nutrition. Nevertheless, it is only in the last 40 years that it has captured the interest and imagination of policy makers and the public alike. Today, not only is nutrition shaping the development of new food products, but technological innovations have helped create a range of nutritious and functional foods. This chapter provides basic information relevant to food scientists, technologists and food suppliers.

We require food for four main reasons:

1. as a source of energy;
2. as a source of raw materials for growth and development;
3. to supply minute chemicals that serve to regulate vital metabolic processes;
4. to supply food components (phytochemicals) that retard the development and progression of degenerative diseases.

12.2 Human energy requirements

It is no exaggeration to claim that the science of nutrition was founded on the study of energy metabolism. The largest contribution to energy expenditure is basal metabolic rate (BMR). BMR may be defined as the sum total of the minimum activity of all tissue cells of the body under steady-state conditions. It is

also referred to as the minimal rate of energy expenditure compatible with life.

Although the BMR can be measured using direct calorimetry, its measurement is usually made indirectly. Indirect calorimetry measures the consumption of oxygen, the expiration of carbon dioxide, and the elimination of urinary nitrogen. These may then be used to measure the oxidation of fuel. From the respiratory quotient (RQ), which is the ratio of the carbon dioxide expired to the oxygen inhaled, the amount of heat being produced can be calculated.

12.2.1 Indirect calorimetry

Indirect calorimetry refers to the calculation of heat production using the measurement of gaseous exchange – notably, oxygen consumed and carbon dioxide expired. Whilst the heat equivalent of respiratory exchange is usually calculated from this, it is also dependent on the ratio of the moles of carbon dioxide produced to the moles of oxygen consumed, and this is called the respiratory quotient (RQ):

$$\text{RQ} = \frac{\text{moles } CO_2}{\text{moles } O_2}$$

The RQ varies when carbohydrate (carbohydrates), fat, and protein are oxidized. Differences in their composition determine the amount of oxygen required for complete oxidation. RQ for carbohydrates is 1.0. The amount of molecular oxygen required for oxidation is equal to the carbon dioxide produced during the combustion of carbohydrates. The oxidation of glucose may be illustrated as:

$$C_6H_{12}O6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$$

Fats require more oxygen than carbohydrates for combustion as the fat molecule contains a lower ratio of oxygen to carbon and hydrogen. Thus, for fat, the RQ is represented as:

$$2\,C_{57}H_{110}O_6 + 163\,O_2 \rightarrow 114\,CO_2 + 110\,H_2O$$

$$\frac{CO_2}{O_2} = \frac{114}{163} = 0.70$$

The calculation of the RQ for protein is more complex than that for fat or carbohydrates as protein is not completely oxidized. Both carbon and oxygen are excreted in the urine mainly as urea. When adjustment is made for urinary excretion, the ratio of carbon dioxide produced to oxygen consumed is approximately 1:1.2, producing an RQ of 0.80.

Table 12.1 Non-protein RQ and calorific equivalent for oxygen and carbon dioxide.

Non-protein respiratory quotient	Oxygen		Carbon dioxide	
	kcal/l	kJ/l	kcal/l	kJ/l
0.70	4.686	19.60	6.694	28.01
0.72	4.702	19.67	6.531	27.32
0.74	4.727	19.78	6.388	26.73
0.76	4.732	19.80	6.253	26.16
0.78	4.776	19.98	6.123	25.62
0.80	4.801	20.09	6.001	25.11
0.82	4.825	20.19	5.884	24.62
0.84	4.850	20.29	5.774	24.16
0.86	4.875	20.40	5.669	23.72
0.88	4.900	20.50	5.568	23.30
0.90	4.928	20.62	5.471	22.89
0.92	4.948	20.70	5.378	22.50
0.94	4.973	20.81	5.290	22.13
0.96	4.997	20.91	5.205	21.78
0.98	5.022	21.01	5.124	21.44
1.00	5.047	21.12	5.047	21.12

Table 12.1 shows the non-protein RQ and the calorific equivalent for oxygen and carbon dioxide. The calorie equivalence of oxygen alone is usually used for the estimation of energy expenditure as it varies little within an RQ range of 0.7 to 0.86 (compared to CO_2).

Table 12.2 represents the oxygen consumed, carbon dioxide produced and energy equivalence of oxygen when protein, fat and carbohydrates are metabolized.

12.2.2 Energy expenditure estimation: a shortcut method

Almost 60 years ago, Weir (1949) showed that energy expenditure (E) may be calculated without the need to obtain RQ, as shown in the equation below:

$$\text{E (kJ}^{-1}\text{min)} = \frac{20.58\dot{V}}{100}(20.93 - O_{2e})$$

where

$\dot{V}$ is the volume of expired air in liters per minute at standard temperature and pressure (STP),

O_{2e} is expressed as percentage oxygen content of expired air.

identity of an element is decided by the number of protons in the nucleus and therefore by the number of electrons in the extra nuclear structure.

Isotopes Two atoms of the same element with same number of protons but different number of neutrons in their nucleus are called isotopes. Chlorine (35 and 37), oxygen (16 and 18) and nitrogen (14 and 15) have naturally occurring isotopes, but their concentration of the isotopes in nature is low.

Radioisotopes are the isotopes prepared by the bombardment of natural isotopes with atomic particles. These are unstable isotopes, which emit radiation and atomic particles and change into more stable form. The radiation and particles they emit are extremely harmful to living cells. Thus, radiotherapy is used to destroy the malignant cells of cancer.

Since World War II (1945), atomic explosions from atomic bomb or due to accident in nuclear plants have resulted in production of several radioisotopes of elements. These radioisotopes, when distributed by air currents, found their way into plants and animals and hence into the food. Isotopes of the same element have the same number of electrons and therefore enter into chemical reactions in the body, which are harmful to the body.

There are instrumental methods to measure the isotope concentration. Hence radioactive isotopes have been used in research to study the pathways of biological reactions in the body. Thus, utilization and metabolism of nutrients in the body has been studied by feeding isotopically labelled nutrients to animals. The elements most important in food science are hydrogen, carbon, nitrogen, oxygen, fluorine, sodium, phosphorus, sulphur, chlorine, potassium, calcium, iron and iodine.

Molecules

A molecule is formed by the combination of two or more atoms of an element. It is the smallest particle of an element or compound capable of a free (independent) existence. In forming a molecule, the atoms either share an electron or completely transfer one or more electrons from one atom to another to form the outermost shell. These electrons in the outermost shell are known as **valence electrons**.

Organic Chemistry

You may wonder why a special branch of chemistry, namely, organic chemistry is devoted to carbon compounds. There are two important reasons for this. Firstly carbon compounds are the products of living cells. Secondly, there are many more compounds of carbon known than any other element. In organic chemistry, a single molecular formula may represent different compounds, having different molecular arrangements.

TABLE 2.3 Differences between Inorganic and Organic Compounds

Inorganic compounds	Organic compounds
1. Exist as ions.	Do not exist as ions.
2. Have high melting points.	Have low melting points.
3. No vapour forms on boiling—boiling point is high.	On boiling some molecules escape as vapour. Boiling point is low.
4. Since the atoms exist as ions, they react instantly.	As they are not ionized, they do not react freely with other compounds.
5. A few carbon compounds are inorganic. These include Carbon Monoxide, Carbon Dioxide, Carbon Disulphide, Cyanides, Carbonates and Bicarbonates.	All the remaining carbon compounds are organic compounds which contain hydrogen and often oxygen. In additon, they may contain Nitrogen, Sulphur Phosphorus and the Halogens.

Hydrocarbons

Compounds of carbon and hydrogen are known as **hydrocarbons**. These do not occur in foods and are not nutrients. But it is important to study the chemistry of hydrocarbons as a base, before studying the chemistry of nutrients, which are more complex.

Hydrocarbons exist in nature as straight chain compounds of carbon and hydrogen or as cyclic compounds of carbon and hydrogen. The simplest straight chain compound is methane (CH_4) and cyclic compound is cyclohexane (C_6H_{12}). An important cyclic hydrocarbon which forms a base for several complex compounds occurring in foods is benzene (C_6H_6) in which alternate single and double bonds connect the six carbon atoms to form a hexagon. Ring structures which contain an element other than carbon are known as **heterocyclic compounds**. In nature, one finds a wide range of heterocyclic compounds e.g., vitamins.

TABLE 2.4 Important Classes of Organic Compounds

Class	Name of the functional group	Example
Alcohol	Hydroxyl group (OH)	CH_3OH Methyl Alcohol
Phenol	Hydroxyl group attached to a benzene ring	C_6H_5OH Phenol
Aldehyde	Aldehyde group(CHO)	CH_3CHO Acetaldehyde
		C_6H_5CHO Benzaldehyde
Ketone	Carbonyl group(CO)	CH_3-CO-CH_3 Acetone
Acid	Carboxyl Group(COOH)	CH_3COOH Acetic Acid
		C_6H_5COOH Benzoic Acid

Alcohols and Acids Occurring in Foods

In an alcohol, one of the hydrogen atoms of a hydrocarbon molecule has been replaced by a hydroxyl (OH) group. Most of the acids occurring in foods have a hydrogen atom in a hydrocarbon replaced by a carboxyl (COOH) group.

Alcohols and acids are important classes of organic compounds found in foods.

Alcohols

Simple alcohols are named according to the alkyl group they contain. For example, methyl alcohol (CH_3OH), ethyl alcohol or ethanol (C_2H_5OH), amyl alcohol ($C_5H_{11}OH$), and so on.

Alcohols containing three or more carbon atoms, e.g., propyl alcohol, can have isomers. Thus two propyl alcohols are—n-propyl alcohol ($CH_3CH_2CH_2OH$) and isopropyl alcohol $(CH_3)_2CHOH$.

Alcohols, which contain only one hydroxyl group are called **monohydric alcohols**. Those containing two hydroxyl groups are called **dihydric** and three hydroxyl groups, **trihydric alcohols**. Glycerol, an important constituent of fats and oils, is a trihydric alcohol.

Ethyl Alcohol Ethyl alcohol is referred to as alcohol, as it is a constituent of all alcoholic drinks. Alcoholic drinks are manufactured by the fermentation of fruits (mainly grapes) and molasses (a sugar syrup got as a by-product of sugar manufacture). Other cheap sources used to produce alcohol are barley, rice or roots such as potatoes.

Glycerol Glycerol is a trihydric alcohol, i.e., it contains three hydroxyl groups. It is the only important trihydric alcohol. It is a colourless, sweetish liquid and is also known as **glycerine**. It is an important component of fats and oils and will be discussed in that chapter.

Acids

Acids are the important constituents of foods. Acids have the following properties:

- Acids have a sour taste.
- Acids change the colour of some natural dyes (blue to red).
- Acids react with many metals.
- Acids react with bases to form salts.

Acidity and pH You may have studied the relationship of acidity and pH. Pure water is said to have a neutral pH, i.e., ionization results in production of equal number of hydrogen and hydroxyl ions. Thus, it has a pH of 7.0.

An acidic solution has a hydrogen ion concentration of less than pH 7.0 and an alkaline solution has a pH of more than 7.

A pH change of one is a ten-fold change in hydrogen ion concentration.

Inorganic acids are completely ionized in solution and are called *strong* acids.

Thus, 0.1M HCl has a pH a 1.0

Buffer Systems A buffer system is one that resists change in pH when small amounts of acid or alkali are added to it. A buffer system is formed by a mixture of a weak acid and its salt or a weak base and its salt.

A weak acid or a base is only partially ionized in solution; therefore, there is equilibrium between undissociated acid and its ions. Acetic acid, a component of vinegar, is a typical weak acid. Acetic acid of 0.1M strength is dissociated to only 1 per cent and has a pH of 3.

The pH values of some foods are given in Table 2.5.

TABLE 2.5 pH Values of Some Foods

Food	pH
Lime juice	2.3
Apples	3.0
Orange juice	3.7
Tomatoes	4.3
Potatoes	5.5
Spinach	5.4
Peas	6.0
Evaporated milk	6.0
Butter	6.2

The control of pH by buffers is very important in food processing as also in living bodies.

Carboxylic Acids Most of the acids found in foods are carboxylic acids. Carboxylic acids contain a carboxyl group –COOH. The carboxyl group is a combination of a carbonyl group (C=O) and a hydroxyl group (OH). Acetic acid is a typical carboxylic acid. Carboxylic acids are weak acids with pH between 2.2 to 3.0.

The carboxylic acids starting with acetic acid, are called **fatty acids** because some of the higher members of this series occur in fats in combination with glycerol.

The carboxylic acids important in food chemistry are:

Acetic acid	CH_3COOH
Propionic acid	C_2H_5COOH
Butyric acid	C_3H_7COOH
Caproic acid	$C_5H_{11}COOH$
Caprylic acid	$C_7H_{15}COOH$
Capric acid	$C_9H_{19}COOH$
Lauric acid	$C_{11}H_{23}COOH$
Myristic acid	$C_{13}H_{27}COOH$

Palmitic acid	$C_{15}H_{31}COOH$
Stearic acid	$C_{17}H_{35}COOH$

All the above acids are *saturated fatty acids*, which contain an even number of carbon atoms. The members of the above series, with lower molecular weights are liquids at normal temperatures and are miscible in water.

Acetic Acid is produced when alcohol is oxidized in the presence of a bacterial enzyme.

$$CH_3CH_2OH + O_2 \xrightarrow{\text{Enzyme in } \textit{Acetobacter}} CH_3COOH + H_2O$$

In fact, this is how vinegar, a dilute solution of acetic acid, is produced. The choice of raw material depends on the availability of an inexpensive source. It is malt in U.K., cider in U.S.A. and wine in France. In India, it is molasses. Vinegar is used in pickles and in salads.

Butyric acid is found in sour milk and butter, combined with glycerol. When butter gets rancid, butyric acid is released and gives it the unpleasant rancid smell. Stearic acid is a white wax-like solid, which is insoluble in water.

Unsaturated Acids When oils and fats are hydrolysed, some unsaturated fatty acids are released along with saturated fatty acids. As the name indicates, unsaturated fatty acids contain one or more double bonds. Unsaturated fatty acids have lower melting points than saturated ones containing the same number of carbon atoms. As the proportion of unsaturated fatty acids increases, the softness of fat increases.

Oleic acid, which occurs widely, derives its name from olive oil of which it is the principal acid. Oleic acid contains eighteen carbon atoms. The only double bond in its molecule occurs in the center of the carbon chain. It is called a *mono-unsaturated* fatty acid (MUFA) to indicate it contains only one (mono) double bond. Oleic acid is also the main acid present in human body fat.

It is an interesting fact that the majority of the unsaturated fatty acids obtained from oils and fats contain 18 carbon atoms. Linoleic and linolenic acid occur in linseed oil and hence their names start with "lin". These are two of the commonest unsaturated fatty acids. As you may note from formulas given below, linoleic acid contains two double bonds in positions 6 and 9; linolenic acid contains three double bonds in positions 3, 6 and 9.

Stearic acid, a saturated fatty acid, contains 18 carbon atoms. The differences in the structure of these four fatty acids are shown below:

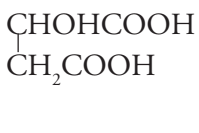
$CH_3(CH_2)_{16}COOH$	Stearic acid
$CH(CH_2)_7$ $CH{=}{=}CH(CH_2)_7$ $COOH$	Oleic acid
$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	Linoleic acid
$CH_3CH_2CH{=}{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	Linolenic acid

Dicarboxylic Acids, as the name indicates contain two carboxylic groups. *Oxalic acid* is the simplest one of these and contains simply two carboxyl groups joined together HOOC-COOH. Small amounts of oxalic acid are present in most fruits and vegetables. However, spinach, beet tops and rhubarb contain very large amounts of oxalic acid as calcium oxalate.

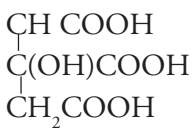
Hydroxy Acids are those acids, which contain both a hydroxyl (OH) and a carboxyl (COOH) group, and these behave both as an alcohol and as an acid. The most important hydroxy acid is *lactic acid* (hydroxy propionic acid). It is produced when milk sugar, lactose is hydrolysed by lactic bacilli to lactic acid. The same chemical change occurs in the preparation of fermented milk products such as curd (yoghurt), butter and cheese.

$$\underset{\text{Lactose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{\text{Enzyme from lactobacilli}} \underset{\text{Lactic acid}}{4CH_3CHOHCOOH}$$

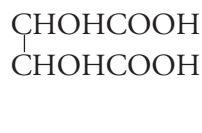
Malic Acid and Citric Acid are two acids commonly present in fruits. Both contain one hydroxyl (OH) group and two and three carboxyl (COOH) groups respectively. Tartaric acid, which contains two hydroxyl and two carboxyl groups, occurs less widely in fruits. Their structures are shown below:



Malic acid



Citric acid



Tartaric acid

Malic and citric acid are responsible for the flavour of fresh fruits. These acids are present in fruits as their potassium salts and also as free acids. Citric acid, as the name suggests, is the main acid of citrus fruits. It is also found in pineapples, tomatoes and most other summer fruits. Malic acid is found in grapes, unripe apples, plums and rhubarb. Tartaric acid is present in grapes and tamarind.

These acids are manufactured commercially for use in beverages, preserves and candies. One of tartaric acid salts is also used in baking powder.

Most of the citric acid and malic acid are absorbed and oxidized in the body; most of the tartaric acid is not absorbed in the body.

Acids act as food preservatives. Acids may be produced in foods during fermentation, or may be added to food to preserve them.

The acids and their salts added to foods lower the pH values and inhibit growth of microorganisms.

Points to Remember

Atoms and Atomic Particles An atom, the smallest particle of an element, is made up of three smaller particles—proton, neutron and electron.

Isotopes and Radio Isotopes Isotopes are the atoms of an element with different number of neutrons in the nucleus; radio isotopes, used in radiotherapy of cancer, are prepared by the bombardment of natural isotopes with atomic particles.

Molecule The smallest particle of an element or compound capable of free independent existence.

Organic Chemistry A special branch of chemistry devoted to the study of carbon compounds, which form the basis of most of the foods.

Alcohols Compounds containing one or more hydroxy groups, e.g., ethyl alcohol a constituent of all alcoholic drinks, glycerol, a trihydric alcohol, which is a constituent of all fats.

Organic Acids Organic acids contain one or more carboxyl groups and are important constituents of many foods, e.g., malic, citric and tartaric acids. Higher members of organic acids occur in fats, in combination with glycerol.

Study Questions

1. What are atoms? What are they made of? Explain the difference between an atom and a molecule.
2. Show diagrammatically the difference between a hydrogen and carbon atom.
3. Why it is important to study organic chemistry?
4. List the differences between inorganic and organic compounds.
5. What are alcohols? Why is it important to study the role of glycerol in foods?
6. Define pH and explain its significance.
7. What are organic acids? What is the difference between a saturated and unsaturated organic acids?
8. List the fatty acids important in foods. Write down their chemical composition.

20.1 Basics of Water Activity

Water is an important constituent of all foods. Why water activity and not water content? In the middle of the nineteenth century, scientists began to discover the existence of a relation between water in a food and its relative tendency to spoil. They also began to realize that the *active water* could be much more important to the stability of food than the total amount of water present. Scott [130,131] clearly identified that water activity of a medium correlated well with the deterioration of food stability due to the growth of microorganism. Thus, it was possible to develop generalized rules or limits for the stability of foods by using water activity. This was the main reason why food scientists started to emphasize water activity rather than water content. Since then, the scientific community has explored the great significance of water activity in determining the physical characteristics, processes, shelf life, and sensory properties of foods. The water activity of fresh foods, as shown by Chirife and Fontan [34], is 0.970–0.996. Other applications of water activity are: (i) process design and control, (ii) ingredient selection, and (iii) packaging selection. Water activity data are important to food processing, such as osmotic dehydration and air drying. In drying operations, desorption isotherms at the process temperature are needed for design and control purposes. The endpoint of drying or osmotic dehydration process can be determined from the equilibrium moisture content. In the drying process, the foods equilibrate with air equilibrium relative humidity; in osmotic or salting process, foods equilibrate with the osmotic solution water activity. Hence, water activity plays an important role in designing, operation, and control of drying processes and reverse osmosis. Water activity's depressing power of solutes needs to be considered when selecting ingredients or additives for food product formulation. When food materials are packed in a semipermeable membrane, the food will (a) collect moisture if its water activity is lower than the external relative humidity of the air or (b) lose moisture if its water activity is higher than the relative humidity. The sorption isotherm is necessary to predict the moisture transfer rate through the packaging film and edible food coating, so that shelf life can be predicted. The mathematical equations used to determine the isotherms for moisture transfer through packaging material are available in the literature [44,114].

20.1.1 Basic Terminologies

A number of basic terminologies related to water activity have been developed over the last 5 decades. It is important to understand these terminologies for proper utilization of water activity concept in food preservation and processing.

20.1.1.1 Water Activity

Water activity, a thermodynamic property, is defined as the ratio of vapor pressure of water in a system and the vapor pressure of pure water at the same temperature, or the equilibrium relative humidity of the air surrounding the system at the same temperature. A number of methods have been reported in literature to measure or estimate the water activity of foods. Water activity measurement methods include the following: (i) equilibrium sorption rate method (isopiestic method), (ii) vapor pressure measurement method, and (iii) hygrometric instrument method. In addition, water activity can be predicted from other thermodynamic properties such as freezing point. The accuracy of most methods lies in the range of 0.01–0.02 water activity units [118]. Details of the various measurement techniques are described by Labuza et al. [80], Rizvi [118], Rahman [109], Rahman and Sablani [112], Rahman et al. [113], Fontana [53], and Sablani et al. [124]. Water activity can be lowered or controlled by several methods such as separating out of water and adding solutes. Processes that can be used to remove water are drying, concentration, and dewatering by centrifuge. Other unit operations such as baking, extrusion, and frying also reduced the water activity to some extent. Solutes can be added to foods to reduce water activity as well as improve the

TABLE 20.1

Some Criteria for Humectants to be Used in Foods

Safe
Approved by regulatory agencies
Effective at reasonable concentrations
Compatible with the nature of the food
Flavorless at concentrations of use
Colorless and imparts no color changes in the food

functional and sensory properties of foods, for example, adding salt to meat and fish, and adding sugars to fruits. When only solutes are used to reduce water activity, then the specific antimicrobial effects and the cost of solutes or humectants should be considered for food product formulation. The factors affecting the selection of humectants are summarized in Table 20.1.

20.1.1.2 Sorption Isotherm

The moisture sorption isotherm is the dependence of moisture content on the water activity of one of the samples at a specified temperature. It is usually presented in a graphical form or as an equation. Brunauer et al. [19] classified adsorption isotherms of materials into five general types (Figure 20.1). If water-soluble crystalline components are present in foods, e.g., sugars or salt, the isotherm appears as concave shape type III. Most other foods result sigmoid isotherm type II. The inflection point of the isotherm indicates the change of water-binding capacity or of the relative amounts of free and bound water. Type I is indicative of a nonswelling porous solid, such as silicate anticaking agents. For practical purposes, the isotherm is presented in an empirical or theoretical model equation. However, none of the isotherm models in the literature is valid over the entire water activity scale of 0–1. The Guggenheim-Anderson-de Boer (GAB) model is one of the most widely accepted models for foods over a wide range of water activities from 0.10 to 0.9. The details of the isotherm models with their parameters are compiled by Rizvi [118], Okos et al. [106], Lomauro et al. [91,92], and Rahman [109].

20.1.1.3 Hysteresis

The difference in the equilibrium moisture content between the adsorption and desorption curves is called hysteresis and is shown in Figure 20.2. In region II of this figure, the water is held less tightly and is usually present in small capillaries, whereas in region III, the water is held loosely in large capillaries or is free [53]. Hysteresis in sorption has important theoretical and practical implications in foods. The theoretical implications are evidence of irreversible of the sorption process and the validity of the equilibrium thermodynamic process. The practical implications deal with the effects of hysteresis on chemical and microbiological deterioration and its importance on low- and intermediate-moisture foods [70]. Strasser [140] and Wolf et al. [154] maintained that changes in hysteresis could be used as an index of quality deterioration, since hysteresis loops of foods change with storage time, but this is a poor method of evaluation. Rahman and Al-Belushi [111] presented more reviews on the sorption hysteresis in foods.

20.1.1.3.1 Factors Affecting Hysteresis

The desorption hysteresis loop usually ends at the monolayer, but in some cases it extends down to an activity of zero [77]. In foods, a variety of hysteresis loop shapes can be observed depending on the type of food and the temperature [152]. The principal factors affecting hysteresis are composition of the product, isotherm temperature, storage time before isotherm measurement, pretreatments, drying temperature, and the number of successive adsorption and desorption cycles.

20.1.1.3.1.1 Types of Foods Affecting Hysteresis Variations in hysteresis can be grouped into three types of foods [70]: (i) Hysteresis in high-sugar foods—in high-sugar or high-pectin foods such as air-dried apple, hysteresis occurs mainly in the monomolecular layer of water region, below the first inflection point of isotherm region I in Figure 20.2 [106]. Although the total hysteresis is large, there is no hysteresis above 0.65. (ii) Hysteresis in high-protein foods—in pork, a moderate hysteresis begins at about

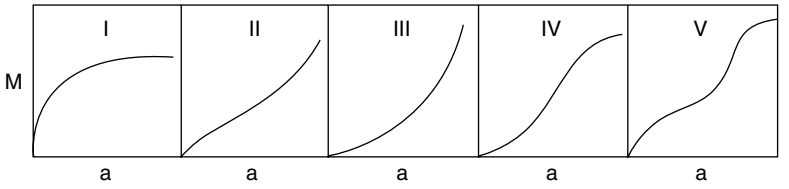


FIGURE 20.1 The five types of van der Waals adsorption isotherms proposed by Brunauer et al. [19].

6.1 INTRODUCTION

Moisture assays can be one of the most important analyses performed on a food product and yet one of the most difficult from which to obtain accurate and precise data. This chapter describes various methods for moisture analysis—their principles, procedures, applications, cautions, advantages, and disadvantages. Water activity measurement also is described, since it parallels the measurement of total moisture as an important stability and quality factor. With an understanding of techniques described, one can apply appropriate moisture analyses to a wide variety of food products.

6.1.1 Importance of Moisture Assay

One of the most fundamental and important analytical procedures that can be performed on a food product is an assay for the amount of moisture (1–3). The dry matter that remains after moisture removal is commonly referred to as **total solids**. This analytical value is of great economic importance to a food manufacturer because water is an inexpensive filler. The following listing gives some examples in which moisture content is important to the food processor.

1. Moisture is a quality factor in the preservation of some products and affects stability in:
   a. dehydrated vegetables and fruits
   b. dried milks
   c. powdered eggs
   d. dehydrated potatoes
   e. spices and herbs.
2. Moisture is used as a quality factor for:
   a. jams and jellies, to prevent sugar crystallization
   b. sugar syrups
   c. prepared cereals—conventional, 4–8%; puffed, 7–8%.
3. Reduced moisture is used for convenience in packaging or shipping of:
   a. concentrated milks
   b. liquid cane sugar (67% solids) and liquid corn sweetener (80% solids)
   c. dehydrated products (these are difficult to package if too high in moisture)
   d. concentrated fruit juices.
4. Moisture (or solids) content is often specified in compositional standards (i.e., Standards of Identity):
   a. Cheddar cheese must be ≤39% moisture.
   b. Enriched flour must be ≤15% moisture.
   c. Pineapple juice must have soluble solids of ≥10.5 °Brix (conditions specified).
   d. Glucose syrup must have ≥70% total solids.
   e. The percentage of added water in processed meats is commonly specified.
5. Computations of the nutritional value of foods require that you know the moisture content.
6. Moisture data are used to express results of other analytical determinations on a uniform basis (i.e., dry weight basis).

6.1.2 Moisture Content of Foods

The moisture content of foods varies greatly, as shown in Table 6-1 (4). Water is a major constituent of most food products. The approximate, expected moisture content of a food can affect the choice of the method of measurement. It also can guide the analyst in determining the practical level of accuracy required when measuring moisture content, relative to other food constituents.

6.1.3 Forms of Water in Foods

The ease of water removal from foods depends on how it exists in the food product. The three states of water in food products are:

1. **Free water**—This water retains its physical properties and thus acts as the dispersing agent for colloids and the solvent for salts.
2. **Adsorbed water**—This water is held tightly or is occluded in cell walls or protoplasm and is held tightly to proteins.
3. **Water of hydration**—This water is bound chemically, for example, lactose monohydrate; also some salts such as $Na_2SO_4 \cdot 10H_2O$.

Depending on the form of the water present in a food, the method used for determining moisture may measure more or less of the moisture present. This is the reason for official methods with stated procedures (5–7). However, several official methods may exist for a particular product. For example, the AOAC International methods for cheese include: Method 926.08, vacuum oven; 948.12, forced draft oven; 977.11, microwave oven; 969.19, distillation (5). Usually, the first method listed by AOAC International is preferred over others in any section.

6.1.4 Sample Collection and Handling

General procedures for sampling, sample handling and storage, and sample preparation are given in Chapter 5. These procedures are perhaps the greatest potential source of error in any analysis. Precautions must be taken to minimize inadvertent **moisture losses or gains** that occur during these steps. Obviously, any exposure of a sample to the open atmosphere should be as short as possible. Any heating of a sample by friction during grinding should be minimized. Headspace in the

6-1 table **Moisture Content of Selected Foods**

Food Item	*Approximate Percent Moisture (Wet Weight Basis)*
Cereals, bread, and pasta	
Wheat flour, whole-grain	10.3
White bread, enriched (wheat flour)	13.4
Corn flakes cereal	1.7–3.5
Crackers saltines	4.1
Macaroni, dry, enriched	10.2
Dairy products	
Milk, whole, fluid, 3.3% fat	88.0
Yogurt, plain, low fat	85.1
Cottage cheese, low fat or 2% milk fat	79.3
Cheddar cheese	36.8
Ice cream, vanilla	61.0
Fats and oils	
Margarine, regular, hard, corn, hydrogenated	15.7
Butter, with salt	15.9
Oil—soybean, salad, or cooking	0
Fruits and vegetables	
Watermelon, raw	91.5
Oranges, raw, California navels	86.8
Apples, raw, with skin	83.9
Grapes, American type, raw	81.3
Raisins	15.4
Cucumbers, with peel, raw	96.0
Potatoes, microwaved, cooked in skin, flesh and skin	72.0
Snap beans, green, raw	90.3
Meat, poultry, and fish	
Beef, ground, extra lean, raw	63.2
Chicken, broilers and fryers, light meat, meat and skin, raw	68.6
Finfish, flatfish (flounder and sole species), raw	79.1
Egg, whole, raw, fresh	75.3
Nuts	
Walnuts, black, dried	4.4
Peanuts, all types, dry roasted with salt	1.6
Peanut butter, smooth style, with salt	1.2
Sweeteners	
Sugar, granulated	0
Sugar, brown	1.6
Honey, strained or extracted	17.1

From USDA Nutrient Database for Standard Reference, with modification. Release 14 (July 2001). http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl

sample storage container should be minimal because moisture is lost from the sample to equilibrate the container environment against the sample. It is critical to control temperature fluctuations since moisture will migrate in a sample to the colder part. To control this potential error, remove the entire sample from the container, reblend quickly, then remove a test portion (8 and 9).

To illustrate the need for optimum efficiency and speed in weighing samples for analysis, Bradley and Vanderwarn (10) showed, using shredded Cheddar cheese (2–3 g in a 5.5-cm aluminum foil pan), that moisture loss within an analytical balance was a straight line function. The rate of loss was related to the relative humidity. At 50% relative humidity, it required only 5 sec to lose 0.01% moisture. This time doubled at 70% humidity, or 0.01% moisture loss in 10 sec. While one might expect a curvilinear loss, the moisture loss was actually linear over a 5-min study interval. These data demonstrate the necessity of absolute control during collection of samples through weighing, before drying.

6.2 OVEN DRYING METHODS

In **oven drying methods**, the sample is heated under specified conditions, and the loss of weight is used to calculate the moisture content of the sample. The amount of moisture determined is highly dependent on the type of oven used, conditions within the oven, and the time and temperature of drying. Various oven methods are approved by AOAC International for determining the amount of moisture in many food products. The methods are simple, and many ovens allow for simultaneous analysis of large numbers of samples. The time required may be from a few minutes to over 24 hr.

6.2.1 General Information

6.2.1.1 Removal of Moisture

Any oven method used to evaporate moisture has as its foundation the fact that the boiling point of water is 100°C; however, this considers only pure water at sea level. Free water is the easiest of the three forms of water to remove. However, if 1 g molecular weight (1 mol) of a solute is dissolved in 1.0 L of water, the boiling point would be raised by 0.512°C. This boiling point elevation continues throughout the moisture removal process as more and more concentration occurs.

Moisture removal is sometimes best achieved in a two-stage process. Liquid products (e.g., juices, milk) are commonly predried over a **steam bath** before drying in an oven. Products such as bread and field dried grain are often air dried, then ground and oven dried, with the moisture content calculated from moisture loss at both air and oven drying steps. Particle size, particle size distribution, sample sizes, and surface area during

maximizing quality control and product standardization in food manufacturing (3,4,6,7).

The characteristics of an ideal method for moisture analysis have been described (6–8). The method should be rapid, applicable to a wide range of food products and ingredients, and safely performable by a nontechnical person after brief training. Furthermore, it should use inexpensive and readily available equipment while demonstrating good accuracy and precision. Unfortunately, each of the various moisture analysis methods has its limitations. Generally, analytical methods for moisture determination are selected for either rapidity or accuracy, although both goals are simultaneously sought (4,6).

The accurate determination of moisture poses numerous challenges despite it often employing relatively simple procedures (3,4,9). The complexity of moisture analysis is largely determined by the chemical composition of the food as well as how the water is held within the food (3,7,9). For example, one major problem is the difficulty of completely separating all of the water from the food sample, resulting in an underestimation of the moisture content. However, harsher conditions to remove all moisture from a food may simultaneously cause decomposition of the product. If such decomposition resulted in the production of water or a loss in sample mass, then the accuracy of the method would be severely in question. Significant loss of volatile compounds from the food is another potential difficulty involved in moisture determination. Understanding these and other limitations, as discussed subsequently, will aid in the selection of an appropriate moisture analysis method.

B. Properties of Water in Foods

As stated previously, water exists in nature as one of three physical states: gas, liquid, and solid. These states are also present in food products to varying degrees, depending on the food's chemical composition and storage temperature. For moisture analysis, however, rather than the physical state of water, it is generally more important to recognize the different types of interactions water can have within a food.

Historically, water inside a food product has been described as existing in two forms: "free" and "bound" (7). However, the concept of "bound" water, due to a variety of definitions, is quite controversial and terminating its use as a term has been suggested (2,10–13). One alternative classification scheme involves three broad types of water: free, adsorbed, and chemically bonded (3,4,9). Some water can exist as "free" water within the intergranular spaces and pores of the food (4). Free water has properties similar to pure water, acts as a dispersing medium for hydrophilic macromolecules to form colloidal solutions, and acts as a solvent for lower molecular weight polar compounds (3,4,9). This type of water is the easiest to remove (9). Water can also be "adsorbed" as a mono- or poly-molecular layer on the internal and external surfaces of the solid components (e.g., carbohydrates and proteins) by molecular forces. This water is closely associated with the adsorbing macromolecular surface via hydrogen bonding, dipole–dipole interactions, ionic forces, and van der Waals forces (4,5). Some water can also exist as chemically bonded water of hydration in compounds such as glucose, lactose, sodium sulfate, and potassium tartrate (3,9). This water held as a component within a crystalline lattice is the most resistant to removal by drying.

Despite concerns about the term "bound" water, it continues to be used and should be discussed with respect to moisture analysis. One definition of "bound" water is the water held in a food that displays significantly different properties (e.g., vapor pressure, binding energy, freezability) from those of "free" water (3,10). For example, water designated as "bound" is resistant to freezing unlike "free" water (3,7,13). Based on this definition of "bound" water, water in dilute polysaccharide gels (e.g., carrageenan) is not necessarily "bound" because although the gel matrix impedes its physical separation, some properties of the water (e.g., vapor pressure) in such a gel are similar to those of pure water (14). Thus, various definitions of "bound" water exist, which lead to different methods of measuring "bound" water and consequently different values for its amount in foods (10,11,13). Another perspective is that the water designated previously as "adsorbed" may be viewed as "bound" because the water is interacting and being held physically by polar and ionic groups within the food. Except for "free" surface water, most water may be considered as "bound" to some degree, keeping in mind differences between physically bound adsorbed water and chemically bonded water of hydration in a crystalline lattice (2,7). For physically bound moisture, the strongest adsorbed moisture is recognized as existing at and below the monolayer moisture value (7,13). Monolayer values are most commonly calculated using the Brunauer–Emmet–Teller or Guggenheim–Anderson–de Boer equations for modeling moisture sorption isotherm data (5). Depending on the type of food, monolayer values generally range from a few per cent to about 10% (wet basis), with

2 Food chemistry

Richard A. Frazier

Key points

- Carbohydrate chemistry: structures, properties and reactions of major monosaccharides, oligosaccharides and polysaccharides in foods.
- Proteins: chemistry of the amino acids and their role in protein structure, a description of the major forces that stabilize protein structure and how they are disrupted during protein denaturation.
- Lipids: structure and nomenclature, polymorphism of triglycerides, oil and fat processing (hydrogenation and interesterification), and lipid oxidation.
- Chemistry of minor components in foods: permitted additives, vitamins and minerals.
- Role of water in foods: water activity, its determination and the importance for microbial growth, chemical reactivity and food texture.
- Physical chemistry of dispersed systems: solutions, lyophilic and lyophobic dispersions, colloidal interactions and the DLVO theory, foams and emulsions.
- Chemical aspects of organoleptic properties of foods.

2.1 Introduction

Food chemistry is a fascinating branch of applied science that combines most of the sub-disciplines of traditional chemistry (organic, inorganic and physical chemistry) together with elements of biochemistry and human physiology. Food chemists attempt to define the composition and properties of food, and understand the chemical changes undergone during production, storage and consumption, and how these might be controlled. Foods are fundamentally biological substances and are highly variable and complex; therefore, food chemistry is a constantly evolving and expanding field of knowledge that underpins other areas of food science and technology. This chapter cannot hope to encompass all of the intricacies and details of food chemistry, but instead attempts to provide an overview of the fundamental areas that constitute this important area of science. To delve deeper, the reader is encouraged to refer to one or more of the excellent texts relating to food chemistry that are listed as further reading at the end of this chapter.

2.2 Carbohydrates

Carbohydrate is the collective name for polyhydroxyaldehydes and polyhydroxyketones, and these

compounds form a major class of biomolecules that perform several functions in vivo, including the storage and transport of energy. Indeed, carbohydrates are the major source of energy in our diet. The name carbohydrate derives from their general empirical formula, which is $(CH_2O)_n$; however, the carbohydrate group contains several derivatives and closely related compounds that do not fit this general empirical formula but are still considered to be carbohydrates. There are three distinct classes of carbohydrates: *monosaccharides* (1 structural unit), *oligosaccharides* (2–10 structural units) and *polysaccharides* (more than 10 structural units).

2.2.1 Monosaccharides

The monosaccharides are also termed *simple sugars*, are given the suffix *-ose* and classified as *aldoses* or *ketoses* depending on whether they contain an aldehyde or ketone group. The most common monosaccharides are either *pentoses* (containing a chain of five carbon atoms) or *hexoses* (containing a chain of six carbon atoms). Each carbon atom carries a hydroxyl group, with the exception of the atom that forms the carbonyl group, which is also known as the *reducing group*.

Simple sugars are *optically active* compounds and can contain several asymmetrical carbon atoms. This leads to the possibility for the formation of multiple *stereoisomers* or *enantiomers* of the same basic structure. To simplify matters, monosaccharides are assigned optical configurations with respect to comparison of their highest numbered asymmetric carbon atom to the configuration of D-glyceraldehyde or L-glyceraldehyde (see Fig. 2.1). By convention, the carbon atoms in the monosaccharide molecule are numbered such that the reducing group carries the lowest possible number; therefore, in aldoses the reducing group carbon is always numbered 1 and in ketoses the numbering is started from the end of the carbon chain closest to the reducing group. Most naturally occurring monosaccharides belong to the D-series, i.e. their highest numbered carbon has a similar optical configuration to D-glyceraldehyde.

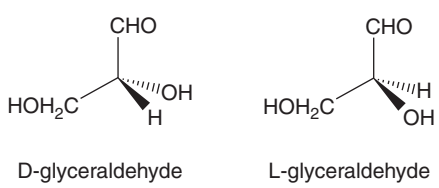


Figure 2.1 The D and L stereoisomers of glyceraldehyde.

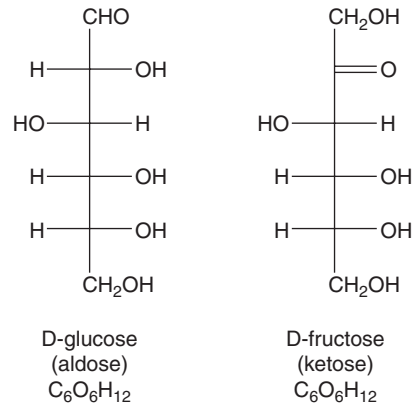


Figure 2.2 Fischer projections of the structures of D-glucose and D-fructose.

The stereochemistry of the monosaccharides is depicted using the *Fischer projection* as shown for D-glucose and D-fructose in Fig. 2.2. All bonds are depicted as horizontal or vertical lines; all horizontal bonds project toward the viewer, while vertical bonds project away from the viewer. The carbon chain is depicted vertically with the C1 carbon at the top.

Aldoses and ketoses commonly exist in equilibrium between their *open-chain* form and cyclic structures in aqueous solution. Cyclic structures form through either a *hemiacetal* or a *hemiketal* linkage between the reducing group and an alcohol group of the same sugar. In this way sugars form either a five-membered *furanose* ring or a six-membered *pyranose* ring as shown in Fig. 2.3 for D-glucose. The formation of a furanose or pyranose introduces an additional asymmetric carbon; hence two *anomers* are formed (α-anomer and β-anomer) from each distinct open-chain monosaccharide. The interconversion between these two anomers is called *mutarotation*.

The cyclic structures of carbohydrates are commonly shown as *Haworth projections* to depict their three-dimensional structure. However, this projection does not account for the tetrahedral geometry of carbon. This is most significant for the six-membered pyranose ring, which may adopt either a *chair* or a *boat* conformation as depicted in Fig. 2.4. Of these, the chair conformation is favoured due to its greater thermodynamic stability. Within this conformation the bulky CH_2OH group is usually found in an equatorial position to reduce steric interactions.

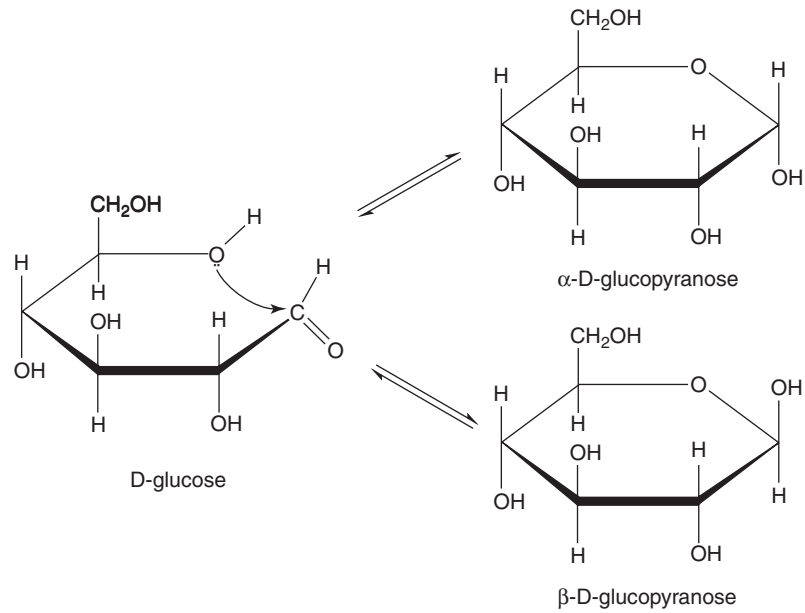


Figure 2.3 The formation of a hemiacetal linkage between the C1 carbon of D-glucose and the hydroxyl group of its C5 carbon leading to two anomers of D-glucopyranose. The rings are depicted as Haworth projections.

2.2.2 Oligosaccharides

Oligosaccharides contain 2–10 sugar units and are water soluble. The most significant types of oligosaccharide occurring in foods are disaccharides, which are formed by the condensation (i.e. water is eliminated) of two monosaccharide units to form a *glycosidic bond*. A glycosidic bond is that between the hemiacetal group of a saccharide and the hydroxyl group of another compound, which may or may not be itself a saccharide. Disaccharides can be homogeneous or heterogeneous and fall into two types:

1 *Non-reducing sugars* in which the monosaccharide units are joined by a glycosidic bond formed between their reducing groups (e.g. sucrose and trehalose). This inhibits further bonding to other saccharide units.

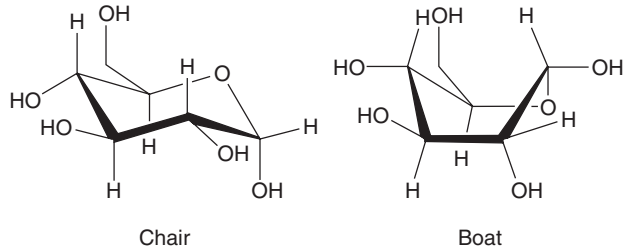


Figure 2.4 Chair and boat conformations of α-D-glucopyranose.

2 *Reducing sugars* in which the glycosidic bond links the reducing group of one monosaccharide unit to the non-reducing alcoholic hydroxyl of the second monosaccharide unit (e.g. lactose and maltose). A reducing sugar is any sugar that, in basic solution, forms an aldehyde or ketone allowing it to act as a reducing agent, and therefore includes all monosaccharides.

Of the disaccharides, sucrose, trehalose and lactose are found free in nature, whereas others are found as *glycosides* (in which a sugar group is bonded through its anomeric carbon to another group, e.g. a phenolic group, via an O-glycosidic bond) or as building blocks for polysaccharides (such as maltose in starch), which can be released by hydrolysis. Probably the three most significant disaccharides in food are sucrose, lactose and maltose, whose structures are depicted in Fig. 2.5.

Sucrose is the substance known commonly in households as sugar and is found in many plant fruits and saps. It is isolated commercially from sugar cane or the roots of sugar beet. Sucrose is composed of an α-D-glucose residue linked to a β-D-fructose residue and is a non-reducing sugar. Its systematic name is α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside (having the suffix *-oside*, because it is a non-reducing sugar). It is the sweetest tasting of the disaccharides and is an important source of energy.

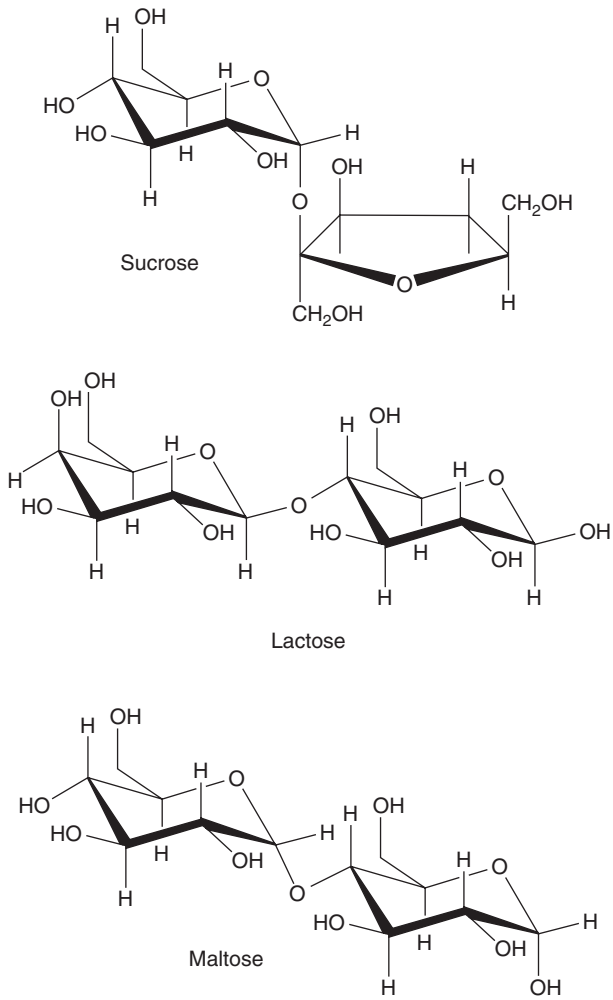


Figure 2.5 The structures of some common disaccharides: sucrose, lactose and maltose.

Lactose is found in mammalian milk and its systematic name is β-D-galactopyranosyl-(1↔4)-β-D-glucopyranose. To aid the digestion of lactose, the intestinal villi of infant mammals secrete an enzyme called lactase (β-D-galactosidase), which cleaves the molecule into its two subunits β-D-glucose and β-D-galactose. In most mammals the production of lactase gradually reduces with maturity into adulthood, leading to the inability to digest lactose and so-called *lactose intolerance*. However, in cultures where cattle, goats and sheep are milked for food there has evolved a gene for lifelong lactase production.

Maltose is formed by the enzymatic hydrolysis of starch and is an important component of the barley malt used to brew beer. It is a homogeneous disaccharide consisting of two units of glucose joined with an α(1→4) linkage, and is systematically named 4-O-α-D-glucopyranosyl-D-glucose. Maltose is a reducing sugar and the addition of further glucose unit yields a series of oligosaccharides known as maltodextrins or simply dextrins.

2.2.3 Polysaccharides

Polysaccharides are built of repeat units of monosaccharides and are systematically named with the suffix *-an*. The generic name for polysaccharides is *glycan* and these can be *homoglycans* consisting of the one type of monosaccharide or *heteroglycans* consisting of two or more types of monosaccharide.

Polysaccharides have three main functions in both animals and plants: as sources of energy, as structural components of cells, and as water-binders. Plant and animal cells store energy in the form of *glucans*, which are polymers of glucose such as starch (in plants) and glycogen (in animals). The most abundant structural polysaccharide is cellulose, which is also a glucan and is found in plants. Water-binding substances in plants include agar, pectin and alginate.

Polysaccharides occur as several structural types: *linear* (e.g. amylose, cellulose), *branched* (e.g. amylopectin, glycogen), *interrupted* (e.g. pectin), *block* (e.g. alginate) or *alternate repeat* (e.g. agar, carrageenan). According to the geometry of the glycosidic linkages, polysaccharide chains can form various conformations, such as *disordered random coil*, *extended ribbons*, *buckled ribbons* or *helices*. One of the most important properties of a great number of polysaccharides in foods is that they are able to form aqueous gels and thereby contribute to food structure and textural properties (e.g. mouth-feel).

2.2.3.1 Starch

Starch occurs in the form of semi-crystalline granules ranging in size from 2 to 100 μm, and consists of two types of glucan: *amylose* and *amylopectin*. Amylose is a linear polymer of α(1→4) linked α-D-glucopyranose and constitutes 20–25% of most starches. Amylopectin is a randomly branched polymer of α-D-glucopyranose consisting of linear chains with α(1→4) linkages with 4–5% of glucose units also being involved in α(1→6) linked branches. On average the length of linear chains in amylopectin is about 20–25 units. The chemical structures of amylose and amylopectin are shown in Fig. 2.6.

Amylose molecules contain in the region of 10^3 glucose units and form helix structures which entrap

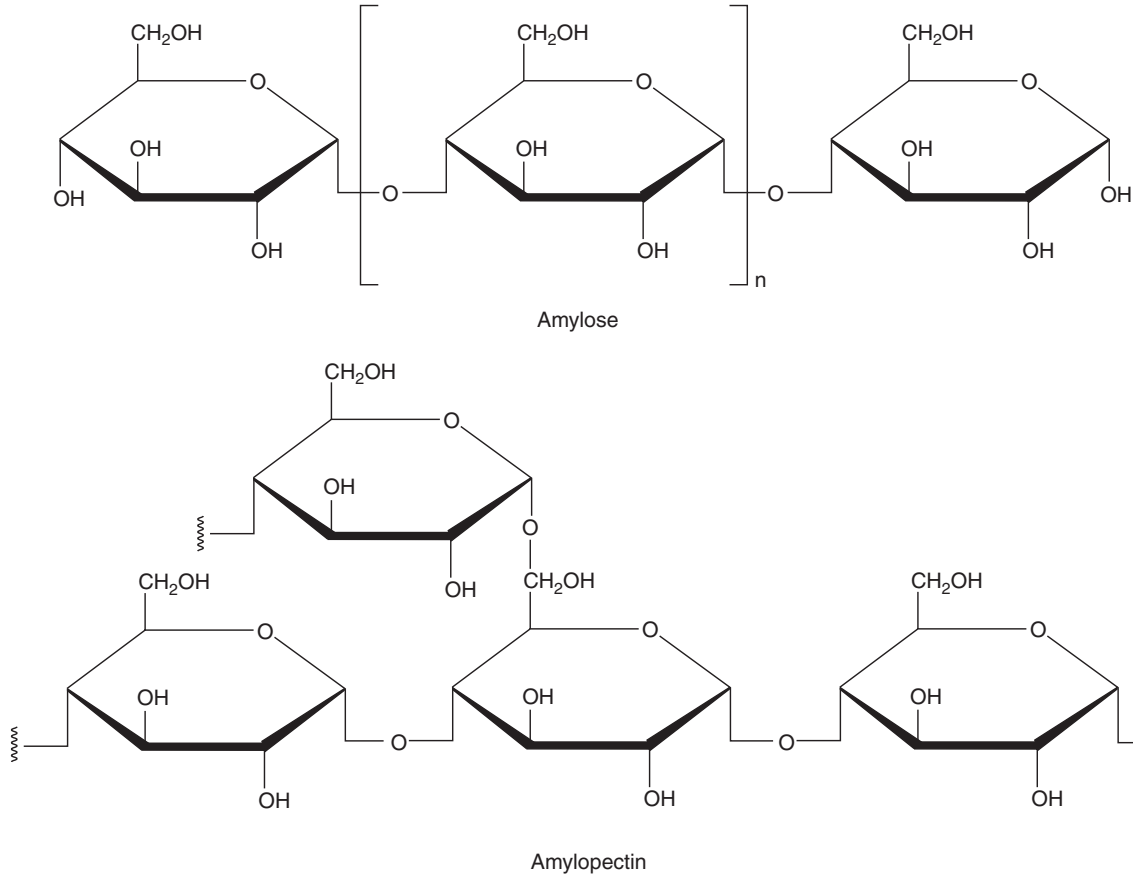


Figure 2.6 Chemical structures of amylose and amylopectin.

other molecules such as organic alcohols or fatty acids to form clathrates or helical inclusion compounds. Indeed, the blue colour that results when iodine solution is used to test for starch is thought to be due to the formation of an inclusion compound.

Amylopectin is a much larger molecule than amylose, containing approximately 10^6 glucose units per molecule, and forms a complex structure. This structure is described by the *cluster model* and has three types of chain (see Fig. 2.7): *A chains* that are unbranched and contain only α(1→4) linkages, *B chains* that contain α(1→4) and α(1→6) linkages, and *C chains* that contain α(1→4) and α(1→6) linkages plus a reducing group. The linear A chains in this structure form clusters that are crystalline in nature, whereas the branched B chains give amorphous regions.

Starch granules undergo a process called *gelatinization* if heated above their gelatinization temperature (55–70°C depending on the starch source) in the presence of water. During gelatinization granules first begin to imbibe water and swell, and as a consequence they progressively lose their organized structure (detected as a loss of *birefringence*). As time

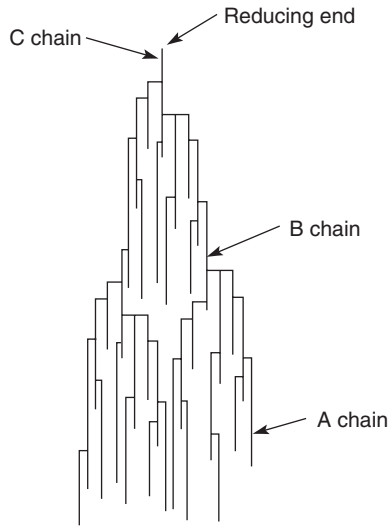


Figure 2.7 Amylopectin cluster model.

progresses, granules become increasingly permeable to water and solutes, thus swelling further and causing the viscosity of the aqueous suspension to increase sharply. Swollen starch granules leach amylose, which further increases viscosity to the extent that a paste is formed. As this paste is allowed to cool, hydrogen-bonding interactions between amylopectin and amylose lead to the formation of a gel-like structure.

Prolonged storage of a starch gel leads to the onset of a process termed *retrogradation*, during which amylose molecules associate together to form crystalline aggregates and starch gels undergo shrinkage and syneresis. Retrogradation can be viewed as a return from a solvated, dispersed, amorphous state to an insoluble, aggregated or crystalline condition. To avoid retrogradation in food products, *waxy starches* can be used that contain only amylopectin. Chemically modified starches are also available that have been depolymerized (i.e. partially hydrolysed), esterified or crosslinked to tailor their properties for particular end uses.

2.2.3.2 Glycogen

The polysaccharide that animals use for the short-term storage of food energy in the liver and muscles is known as *glycogen*. Glycogen is similar in structure to amylopectin, but has much higher molecular weight and a higher degree of branching. Branching aids the rapid release of glucose since the enzymes that release glucose attack on the non-reducing ends, cleaving one glucose molecule at a time. More branching equates to more non-reducing ends meaning more rapid release of energy. The metabolism of glycogen continues post-mortem, which means that by the time meat reaches the consumer it has lost all of its glycogen.

2.2.3.3 Cellulose

The most abundant structural polysaccharide is *cellulose*. Indeed, there is so much cellulose in the cell walls of plants that it is the most abundant of all biological molecules. Cellulose is a linear polymer of β(1→4) linked glucopyranose residues. The β-linkage in cellulose is not susceptible to attack by salivary amylases that break down starch α-linkages, and therefore cellulose forms a major part of *dietary fibre*. Dietary fibre is not digested by enzymes in the small intestine and is hence utilized by colonic microflora via fermentation processes. So-called *hemicelluloses*, including *xylans*, which are major constituents of cereal bran, are another major component of dietary fibre.

2.2.3.4 Pectins

Pectins are mainly used in food as gelling agents. Pectins are heteroglycans and have complex structures that are based on a polygalacturonan backbone of α(1→4)-linked D-galacturonic acid residues, some of which are methylated. Into this backbone, there are regions where D-galacturonic acid is replaced by L-rhamnose, bonded via (1→2) linkages to give an overall rhamnogalacturonan chain. Pectins are characterized by *smooth regions* that are free of L-rhamnose residues and *hairy regions* consisting of both D-galacturonic acid and L-rhamnose residues. The hairy regions are so-called because they carry side chains of neutral sugars including mainly D-galactose, L-arabinose and D-xylose, with the types and proportions of neutral sugars varying with the origin of pectin.

As stated above, pectins are mainly applied in foods for their gelling properties, especially in jams and preserves. *Gels* consist of a three-dimensional polymeric network of chains that entrap water. Pectin gels are stabilized by *junction zones*, which are crystalline regions where smooth regions align themselves and interact. The hairy regions of pectin disrupt these junction zones, preventing extensive aggregation that could lead to precipitation as occurs during amylose retrogradation.

2.2.3.5 Gums

Distinct from those polysaccharides that form gels are a group of polysaccharides that are called *gums*. Gums have a high affinity for water and give high-viscosity aqueous solutions, but are not able to form gels. The reason for this is that all gums possess structures that incorporate a very high degree of branching or highly interrupted chains. This prevents the formation of junction zones (such as in pectins) that are a feature of polysaccharide gels. A notable gum that is commonly employed in foods is xanthan gum, which is secreted by *Xanthomonas campestris* and has a backbone of β(1→4)-linked glucopyranose with trisaccharide branch points every five residues.

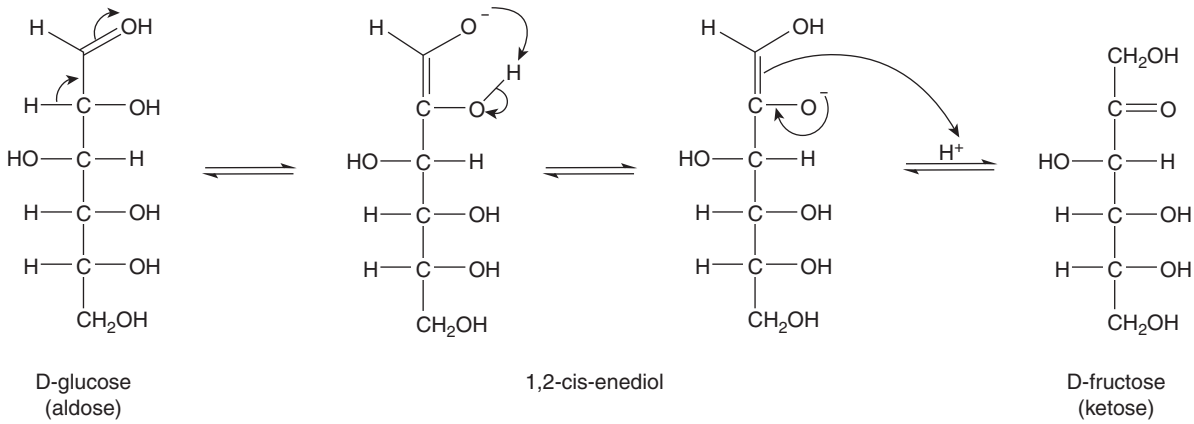


Figure 2.8 The isomerization of sugars to form an enediol intermediate prior to caramelization.

2.2.4 Reactions of carbohydrates

2.2.4.1 Caramelization

When a concentrated solution of sugars is heated to temperatures above 100°C, various thermal decomposition reactions can occur leading to formation of flavour compounds and brown-coloured products. This process, which particularly occurs during the melting of sugars, is called *caramelization*. Caramelization is a *non-enzymic browning reaction* like the Maillard reaction discussed below.

During caramelization, the first reaction step is the reversible isomerization of aldoses or ketoses in their open chain forms to form an *enediol* intermediate (Fig. 2.8). This intermediate can then dehydrate to form a series of degradation products – in the case of hexoses the main product is *5-hydroxymethyl-2-furaldehyde* (HMF), whereas pentoses yield mainly *2-furaldehyde* (furfural). HMF and furfural are considered useful indicators of accurate storage temperature of food samples.

2.2.4.2 Maillard browning

The *Maillard reaction* is the chemical reaction between an amino acid and a reducing sugar that, via the formation of a pool of reactive intermediates, leads to the formation of flavour compounds and melanoidin pigments (non-enzymic browning). The initial step in this complex series and network of reactions is the condensation of reducing sugar and amino acid. The reactive carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid to form an *Amadori compound* as shown in Fig. 2.9. This

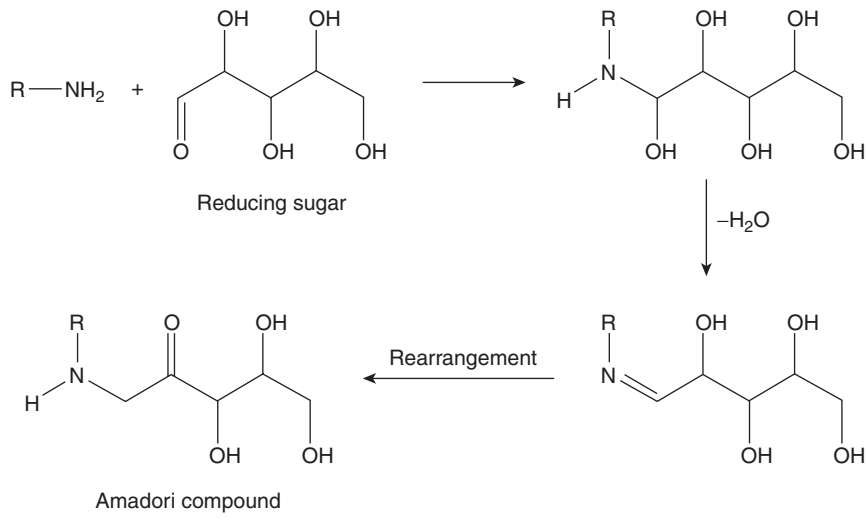


Figure 2.9 The formation of an Amadori compound during the initial stage of the Maillard reaction.

reaction normally requires heat (usually >100°C), is promoted by low moisture content, and is accelerated in an alkaline environment as the amino groups are deprotonated and hence have an increased nucleophilicity. Various reducing sugars have differing rates of reaction in the Maillard reaction; pentoses such as ribose, xylose and arabinose are more reactive than hexoses such as glucose, fructose and galactose. Different sugars give different breakdown products and hence unique flavour and colour.

2.2.4.3 Toxic sugar derivatives

The Maillard reaction, while desirable in many respects, does have certain implications for the loss of essential amino acids (cysteine and methionine), the formation of mutagenic compounds and the formation of compounds that can cause protein cross-linking, which is implicated in diabetes. The most concerning aspect is the potential for toxic sugar derivatives with mutagenic properties, primarily the group of compounds called *heterocyclic amines*. These are particularly associated with cooked meat, especially that which has been grilled at high temperature for long cooking times. In recent times the formation of *acrylamide* has been an issue of concern in potato-based snack foods.

2.3 Proteins

Proteins are polymers of amino acids linked together by peptide bonds. They can also be referred to as *polypeptides*. Proteins are key constituents of food, contributing towards organoleptic properties (particularly texture) and nutritive value. Proteins participate in tissue building and are therefore abundant in muscle and plant tissues.

2.3.1 Amino acids – the building blocks of proteins

2.3.1.1 Amino acid structure

The general structure of an *amino acid* is depicted in Fig. 2.10, and consists of an amino group (NH_2), a carboxyl group (COOH), a hydrogen atom and a distinctive R group all bonded to a single carbon atom, called the α-*carbon*. The R group is called the *side chain* and determines the identity of the amino acid.

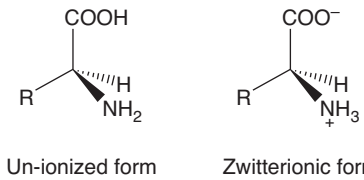


Figure 2.10 The general structure of an amino acid.

Amino acids in solution at neutral pH are predominantly *zwitterions*. The ionization state varies with pH: at acidic pH, the carboxyl group is un-ionized and the amino group is ionized; at alkaline pH, the carboxyl group is ionized and the amino group is un-ionized.

There are 20 different amino acids that are commonly found in proteins. The R group is different in each case and can be classified according to several criteria into four main types: *basic*, *non-polar* (hydrophobic), *polar* (uncharged) and *acidic*. Tables 2.1, 2.2 and 2.3 categorize the amino acids according to these types. The four different functional groups of amino acids are arranged in a tetrahedral array around the α-carbon atom; therefore, all amino acids are optically active apart from glycine. Of the possible L- or D-isomers, proteins contain only L-isomers of amino acids.

Some proteins contain *non-standard amino acids* in addition to the 20 standard amino acids (Fig. 2.11). These are formed by modification of a standard amino acid following its incorporation into the polypeptide chain (*post-translational modification*). Two examples that are encountered often in food proteins are *hydroxyproline* and *O-phosphoserine*. Hydroxyproline occurs in collagen and O-phosphoserine occurs in caseins.

2.3.1.2 Peptide bonds

The *peptide bond* is the covalent bond between amino acids that links them to form peptides and polypeptides (Fig. 2.12). A peptide bond is formed between the α-carboxyl group and the α-amino group of two amino acids by a condensation (or dehydration synthesis) reaction with the loss of water. Peptides are compounds formed by linking small numbers of amino acids (up to 50). A polypeptide is a chain of 50–100 amino acid residues. A protein is a polypeptide chain of 100+ amino acid residues and has a positively charged nitrogen-containing amino group at one end (*N-terminus*) and a negatively charged carboxyl group at its other end (*C-terminus*).

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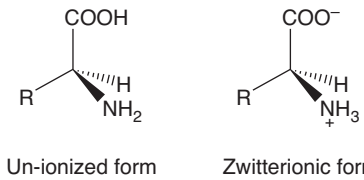

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Table 2.1 Basic and acidic amino acids.

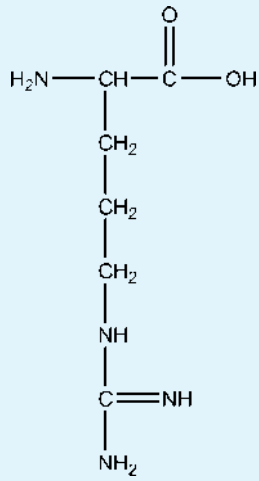
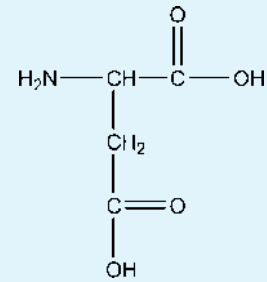
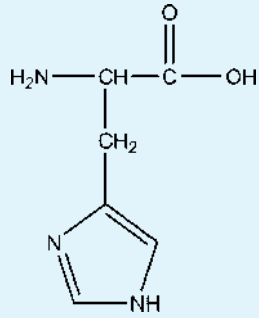
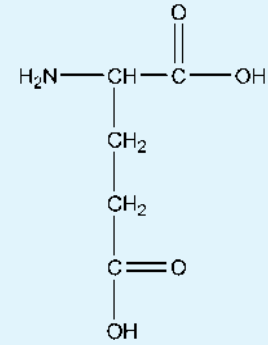
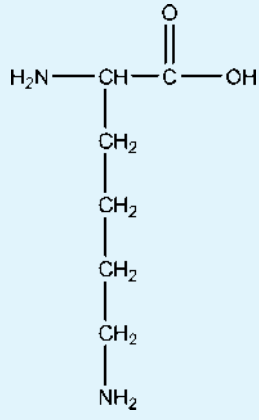
Amino acid	Single letter code	Structural formula	Amino acid	Single letter code	Structural formula
Basic:			**Acidic:**		
Arginine (Arg)	R		Aspartic acid (Asp)	D	
Histidine (His)	H		Glutamic acid (Glu)	E	
Lysine (Lys)	K				

Table 2.2 Non-polar amino acids.

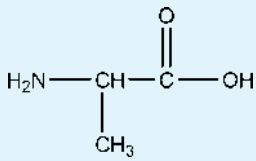
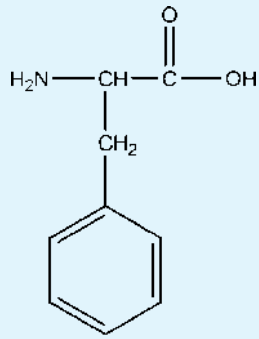
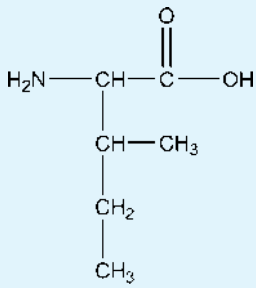
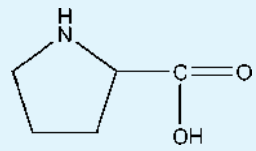
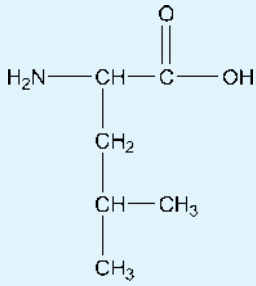
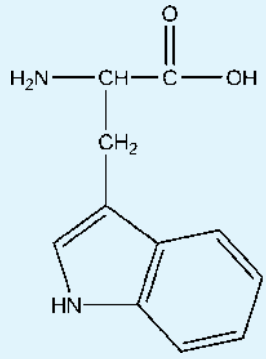
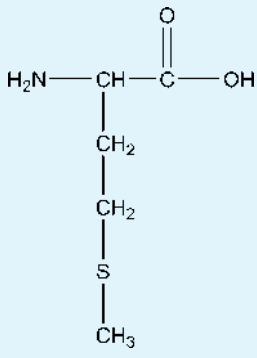
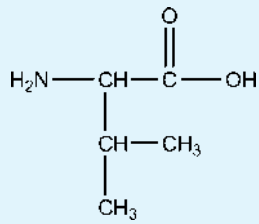
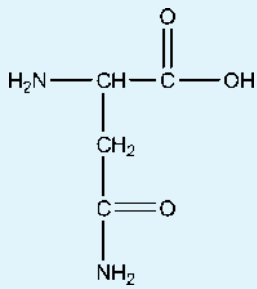
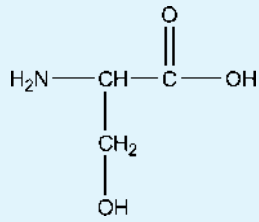
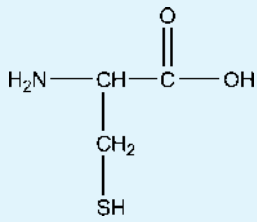
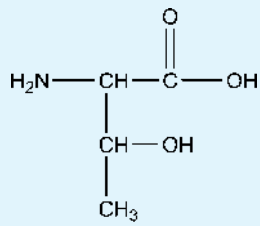
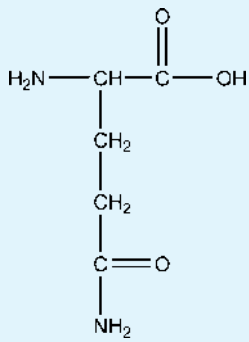
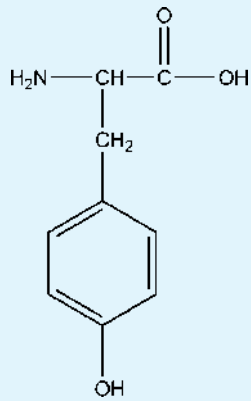
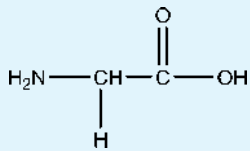
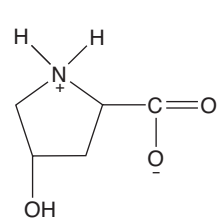
Amino acid	Single letter code	Structural formula	Amino acid	Single letter code	Structural formula
Alanine (Ala)	A		Phenylalanine (Phe)	F	
Isoleucine (Ile)	I		Proline (Pro)	P	
Leucine (Leu)	L		Tryptophan (Trp)	W	
Methionine (Met)	M		Valine (Val)	V	

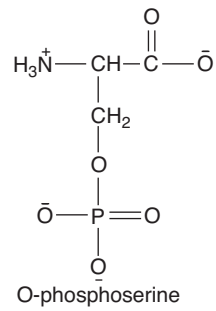
Table 2.3 Polar amino acids.

Amino acid	Single letter code	Structural formula	Amino acid	Single letter code	Structural formula
Asparagine (Asn)	N		Serine (Ser)	S	
Cysteine (Cys)	C		Threonine (Thr)	T	
Glutamine (Gln)	Q		Tyrosine (Tyr)	Y	
Glycine (Gly)	G				

A special feature of the peptide bond is its *partial double bond character*. This arises because the peptide bond is stabilized by *resonance hybridization* between two structures, one single bonded between the carbon and nitrogen atoms, the other double bonded. As a consequence, the peptide bond is planar and stable. This has implications for the possible conformations adopted by a polypeptide chain, since no rotation is possible around the peptide bond. However, rotation is possible around bonds between the α-carbons and the amino nitrogen and carbonyl carbon of their residue.



Hydroxyproline



O-phosphoserine

Figure 2.11 Chemical structures of some non-standard amino acids common in food proteins.

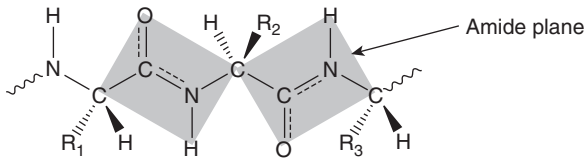


Figure 2.12 Peptide bonds in a polypeptide. The partial double bond character is represented by the dashed double bonds. The shaded boxes highlight atoms that exist within the same plane.

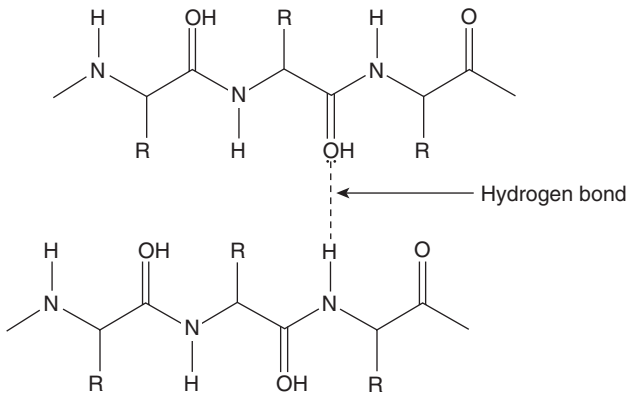


Figure 2.13 Hydrogen bonding between two polypeptides.

2.3.2 Molecular structure of proteins

2.3.2.1 Primary structure

The *primary structure* of a protein is simply the sequence of amino acids listed from the N-terminal amino acid. There are more than a billion possible sequences of the 20 amino acids and every protein will have a unique primary structure which determines how the protein folds into a three-dimensional conformation. If we compare the primary sequence Leu-Val-Phe-Gly-Arg-Cys-Glu-Leu-Ala-Ala with Gly-Leu-Arg-Phe-Cys-Val-Ala-Glu-Ala-Leu, these two peptides have the same number of amino acids, the same kinds of amino acids, but have different primary structures.

2.3.2.2 Secondary structure

The secondary structure of a protein describes the arrangement of the protein backbone (polypeptide chain) due to *hydrogen bonding* between its amino acid residues. Hydrogen bonding can occur between an amide hydrogen atom and a lone pair of electrons on a carbonyl oxygen atom, as shown in Fig. 2.13.

The peptide bond is planar, offering no rotation around its axis. This leaves only two bonds within each amino acid residue that have free rotation, namely the α-carbon to amino nitrogen and α-carbon to carboxyl carbon bonds. The rotations around these bonds are represented by the *dihedral angles* ϕ (phi) and ψ (psi), as shown for a tripeptide of alanine in Fig. 2.14. Ramachandran plotted ϕ and ψ combinations from known protein structures and found that there are certain sterically favourable combinations that form the basis for the preferred secondary structures. He also found that unfavourable orbital overlap precludes some combinations: $\phi = 0^\circ$ and $\psi = 180^\circ$; $\phi = 180^\circ$ and $\psi = 0^\circ$; $\phi = 0^\circ$ and $\psi = 0^\circ$.

Two kinds of hydrogen bonded secondary structures occur frequently with features that repeat at regular intervals. These *periodic structures* are the α-*helix* and the β-*pleated sheet*. The β-*pleated sheet* can give a two-dimensional array and can involve more than one polypeptide chain.

α-Helix

The α-helix is a coiled rod-like structure and involves a single polypeptide chain. The 'α' denotes that if you were to view the helix down its axis then you would note that it spirals clockwise away from you. The α-helix is stabilized by hydrogen bonding parallel to the helix axis and the carbonyl group of each residue is hydrogen bonded to the amide group of the residue that is four residues away if counting from the N-terminus. There are 3.6 residues for each turn of the helix and the dihedral angles are $\phi = -57^\circ$ and $\psi = -48^\circ$. The R group of each residue protrudes from the helix and plays no role in the formation of

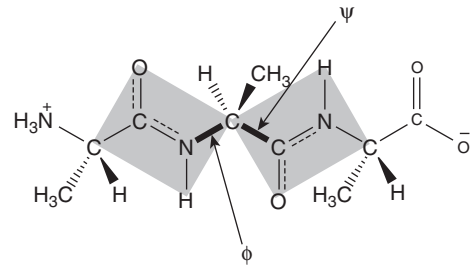


Figure 2.14 Bonds adjacent to peptide bonds with free rotation are depicted in bold with their respective dihedral angles ϕ and ψ.

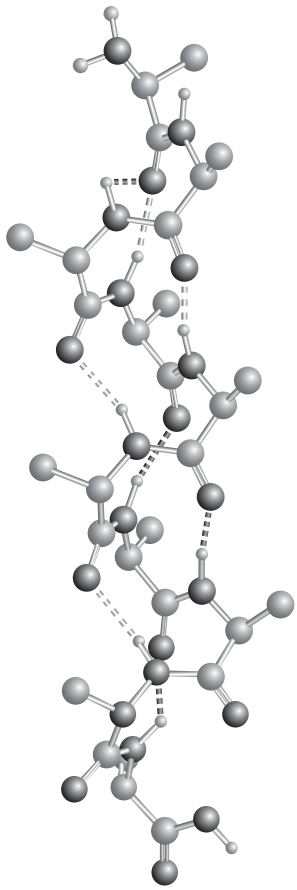


Figure 2.15 Ball and stick representation of an α-helix structure showing the position of hydrogen bonds between amide hydrogens (small white spheres) and carbonyl oxygens (larger dark spheres).

hydrogen bonding as part of the α-helix structure. In the illustration of an α-helix structure shown in Fig. 2.15, the hydrogen bonds are shown as dotted lines between backbone amide hydrogens and backbone carbonyl oxygens.

Proteins contain varying amounts of α-helix structure. Properties of α-helices include strength and low solubility in water. These properties arise because all amide hydrogen and carbonyl oxygen is involved in hydrogen bonds. Multiple strands of α-helix may entwine to make a protofibril such as in the muscle protein myosin.

All amino acids can be found in α-helix structure apart from *proline*, which disrupts α-helix. This is because its cyclic structure causes a bend in the backbone as a result of the restricted C–N bond rotation. This prevents the α-amino group from participating in intrachain hydrogen bonding.

β-*Pleated sheet*

In β-pleated sheets the peptide backbone is almost completely extended (termed a β-*strand*) and hydrogen bonding is perpendicular to the direction of the polypeptide chain. Hydrogen bonds form between different parts of a single chain that is doubled back on itself (*intrachain bonds*) or between different chains (*interchain bond*) giving rise to a repeated zigzag structure (see Fig. 2.16). β-Sheets can be either *parallel* (where the β-strands run in the same direction) or *antiparallel* (where the β-strands run in opposite directions). The dihedral angles are $\phi = -119^\circ$ and $\psi = +113^\circ$ for pure parallel, and $\phi = -139^\circ$ and $\psi = +135^\circ$ for pure antiparallel β-sheets.

β-*Turns*

β-*Turns* are essentially hairpin turns in the polypeptide chain that allow it to reverse direction. In a β-turn the carbonyl oxygen of one residue is hydrogen bonded to the amide proton of a residue three

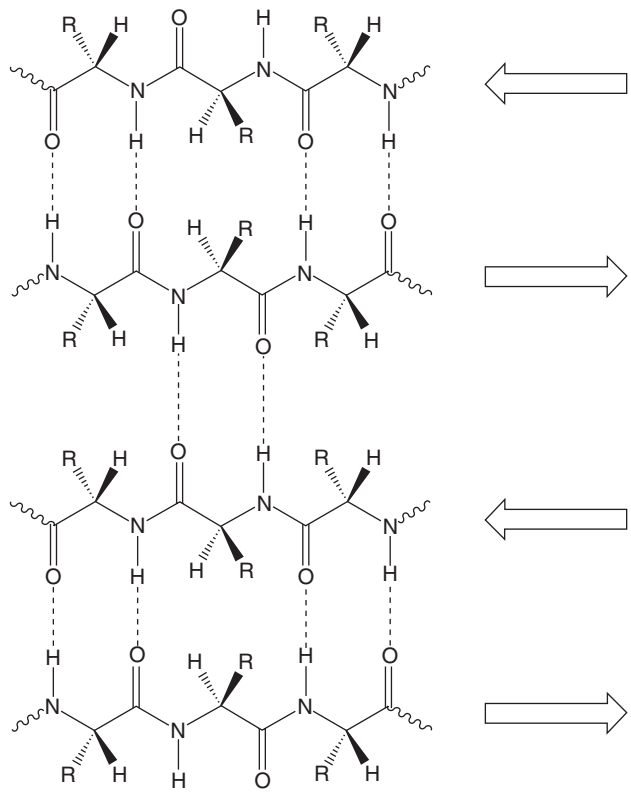


Figure 2.16 Hydrogen bonding within an antiparallel β-sheet structure. The arrow shows the direction of the polypeptide chain.

residues away. Proline and glycine are prevalent in β-turns. β-Turns are estimated to comprise between a quarter and a third of all residues in proteins and they are commonly found to link two strands of anti-parallel β-sheet.

Collagen triple helix

Collagen is a component of bone and connective tissue and is organized as strong water-insoluble fibres. It has a unique periodic structure comprising of three polypeptide chains wrapped around each other in a repeat sequence of X-Pro-Gly or X-Hyp-Gly (where X can be any amino acid). Proline and hydroxyproline make up to 30% of the residues in collagen, and hydroxylysine is also present. Every third position in the *collagen triple helix* is Gly because every third residue must sit inside the helix and only Gly is small enough.

The individual collagen chains are also helices and the three strands are held together by hydrogen bonds involving hydroxyproline and hydroxylysine residues. The molecular weight of the triple-stranded array is approximately 300,000 Daltons, involving approximately 800 amino acid residues. Intra- and intermolecular cross-linking stabilizes the collagen triple helix structure, especially covalent bonds between lysine and histidine. The amount of cross-linking increases with age. A major role for vitamin C (L-ascorbic acid) in vivo is in making collagen: proline and lysine in collagen are converted to 4-hydroxyproline and 5-hydroxylysine using this vitamin. Scurvy is a disease arising from a deficiency of vitamin C, and results in skin lesions, bleeding gums and fragile blood vessels.

2.3.2.3 Tertiary structure

The *tertiary structure* of a protein is the three-dimensional arrangement of all atoms within the molecule, and takes into account the conformations of side chains and the arrangement of helical and pleated sheet sections with respect to each other. Proteins *fold* to make the most stable structure and this structure will generally minimize solvent contact with residues of opposing polarity and hence minimize overall free energy. Therefore, in aqueous solution, proteins generally exist with their hydrophobic residues to the inside and their hydrophilic residues to the outside of their three-dimensional conformation.

There are two categories of tertiary structures:

- *Fibrous proteins* – overall shape is a long rod; mechanically strong; usually play a structural role in nature; relatively insoluble in water and unaffected by moderate changes in temperature and pH.
- *Globular proteins* – helical and pleated sheet sections fold back on each other; interactions between side chains important for protein folding; polar residues face surface and interact with solvent; non-polar residues face interior and interact with each other; structure is not static; generally more sensitive to temperature and pH change than their fibrous counterparts.

The tertiary structure of a protein is held together by interactions between the side chains. These can be through *non-covalent* interactions or *covalent* bonds. The most common non-covalent interactions are *electrostatic* (ionic bonds, salt bridges, ion pairing), *hydrogen bonds*, *hydrophobic interactions* and *van der Waals dispersion forces*. Covalent bonds in protein structure are primarily *disulphide bonds* (sulphur bridges) between cysteine residues, although other types of covalent bond can form between residues.

Electrostatic interactions

Some amino acids contain an extra carboxyl group (aspartic acid and glutamic acid) or an extra amino group (lysine, arginine, histidine). These groups can be ionized and therefore an ionic bond could be formed between the negative and the positive group if the chains folded in such a way that they were close to each other.

Hydrogen bonds

Hydrogen bonds can form between side chains since many amino acids contain groups in their side chains which have a hydrogen atom attached to either an oxygen or a nitrogen atom. This is a classic situation where hydrogen bonding can occur. For example, the amino acid serine contains a hydroxyl group in its side chain; therefore, hydrogen bonding could occur between two serine residues in different parts of a folded chain.

Hydrophobic interactions

Non-polar molecules or groups tend to cluster together in water; these associations are called hydrophobic interactions. The driving force for hydrophobic interactions is not the attraction of the

non-polar molecules for one another, but is due to entropic factors relating to the strength of hydrogen bonding between water molecules.

Van der Waals dispersion forces

Several amino acids have quite large hydrocarbon groups in their side chains (e.g. leucine, isoleucine and phenylalanine). Temporary fluctuating dipoles in one of these groups could induce opposite dipoles in another group on a nearby folded chain. The dispersion forces set up would be enough to hold the folded structure together, although van der Waals forces are weaker and less specific than electrostatic and hydrogen bonds.

Disulphide bonding

If two cysteine side chains are oriented next to each other because of folding in the peptide chain, they can react to form a covalent bond called a *disulphide bond* or a *sulphur bridge*.

2.3.2.4 Quaternary structure

Not all proteins possess *quaternary structure*; it is only a property of proteins that consist of more than one polypeptide chain. Each chain is a subunit of the *oligomer* (protein), which is commonly a dimer, trimer or tetramer. Haemoglobin has quaternary structure. It is a tetramer consisting of two α- and two β-chains. The chains are similar to myoglobin and haemoglobin is able to bind four oxygen atoms through positive cooperativity.

2.3.3 Denaturation of proteins

The forces that stabilize the secondary, tertiary and quaternary structures of proteins can be disrupted through various chemical or physical treatments. This disruption of the native protein structure is defined as protein *denaturation*, which is an important process that may occur during the processing of foods. Denaturation is a change in a protein which causes an alteration in its physical and/or biological properties without rupture of its peptide bonds. It is generally observed as unfolding of the protein molecule from its uniquely ordered structure to a randomly ordered peptide chain. In the case of globular proteins, the denaturing process is often followed by *aggregation*, since previously buried hydrophobic residues are exposed to solution.

Denaturation is accompanied by a loss of native biological activity, but also affects physical properties. Some important consequences of protein denaturation are:

- Loss of biological activity (e.g. enzyme activity).
- Loss of solubility and changes to water-binding capacity.
- Increased intrinsic viscosity.
- Increased susceptibility to proteolysis.

Denaturation can be reversible, but if disulphide bonds are broken the denaturation process is often considered irreversible. Different proteins have different susceptibilities to denaturation since their individual structures are different. There are various denaturing agents that can destabilize protein structures that are categorized as physical agents or chemical agents.

Physical agents include heat, mechanical treatment, hydrostatic pressure, irradiation, and adsorption at interfaces. *Heat* is the most commonly encountered physical agent and is able to destabilize many bonds within proteins, including electrostatic bonds, hydrogen bonds and van der Waals interactions. Heat denaturation is useful in food processing since it tends to lead to improvement of sensory properties and protein digestibility, and can be used to manipulate foaming and emulsifying properties. Heating also promotes the participation of proteins in the Maillard reaction, which leads to the loss of nutritionally available lysine residues.

Chemical agents to denature proteins include acids, alkalis, metals, organic solvents and various organic solutes. Exposure to *acids* or *alkalis* (i.e. pH changes) affects the overall net charge on a protein, which will change the extent of electrostatic interactions, both attractive and repulsive. Most proteins are stable within a pH range around their isoelectric point (zero net charge) and the effects of acids or alkalis are normally reversible.

The presence of *organic solvents* weakens hydrophobic interactions since non-polar side chains become more soluble. *Organic solutes* can have a variety of effects. Urea alters the structure of water in such a way as to weaken hydrophobic interactions, leading to protein unfolding. Sodium dodecyl sulphate (SDS) is an anionic detergent that binds irreversibly to charged groups within a protein, inducing a large net negative charge that increases electrostatic repulsion, leading to unfolding. Reducing

agents, such as mercaptoethanol and dithiothreitol, break disulphide bonds in proteins.

2.3.4 Post-translational modification

Post-translational modification of a protein is a chemical change that has occurred after the protein was synthesized by the body. Post-translational modification can create new functional families of proteins by attachment of biochemical functional groups to reactive groups on amino acid side chains, such as phosphate (*phosphoproteins*), various lipids (*lipoproteins*) and carbohydrates (*glycoproteins*). More simple modifications of amino acid side chains are also possible, such as the hydroxylation of lysine and proline encountered in collagen due to the action of vitamin C.

Enzymes also cause post-translational modification, such as peptide bond cleavage by specific proteases. One example of particular relevance in food production is the action of chymosin on casein. Chymosin cleaves the peptide bond between phenylalanine and methionine in κ-casein, which is used to bring about extensive precipitation and curd formation during cheese making.

2.3.5 Nutritional properties of proteins

Food proteins have an important nutritional role and are primarily used by the body to supply nitrogen and amino acids from which the body synthesizes its own proteins. Within the gastrointestinal tract, hydrolytic enzymes break down food proteins into their component amino acids, which are then used by the body to synthesize other substances. The liver balances the pattern of amino acid supply against the needs of synthesis.

In terms of nutritive value, proteins are classified according to their content of non-essential and essential amino acids. *Non-essential amino acids* are synthesized by the body and require only an adequate supply of amino nitrogen and carbohydrate. However, humans cannot synthesize some *essential amino acids* and these must be supplied by our diet. The essential amino acids are: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

Protein efficiency ratio (PER) is used as a measure of how well food protein sources supply essential amino acids. Human breast milk is treated as a standard and is given a PER score of 100%. In general, animal protein foods (eggs, milk and meat) are very efficient sources, whereas plant protein foods are less efficient since they are usually deficient in either lysine or methionine. For this reason, vegetarians need to consume a balanced diet of plant products to ensure sufficient supply of these two amino acids.

2.4 Lipids

Lipids are a group of molecules that contribute to the structure of living cells and are also used in the body for the purpose of energy storage. Dietary lipids have important roles for provision of energy and as carriers of fat-soluble vitamins. Generally speaking, all lipids are soluble in non-polar organic solvents and have low solubility in water. Dietary lipids are commonly referred to as *oils* and *fats*. Edible oils are liquid at room temperature, whereas fats are solid or semi-solid at room temperature. The lipids found in oils and fats are chemically very diverse, but are predominantly long-chain *fatty acid esters*. Other lipid types encountered in foods are also either fatty acids or derivatives of fatty acids, and include *triglycerides*, *phospholipids*, *sterols* and *tocopherols*.

Lipids can be broadly classified into three main groups:

- *Simple lipids* yield two classes of product when hydrolyzed, e.g. glycerides (acylglycerols) which are hydrolyzed to give glycerol and a fatty acid.
- *Complex lipids* yield three or more classes of product when hydrolyzed, e.g. phospholipids, which are hydrolyzed to give alcohols, fatty acids and phosphoric acid.
- *Derived lipids* are non-hydrolyzable and do not fit into either of the above classes, e.g. sterol, tocopherol and vitamin A.

2.4.1 Lipid structure and nomenclature

2.4.1.1 Fatty acids

A *fatty acid* is a carboxylic acid having a long unbranched aliphatic tail or chain, and can be described chemically as an *aliphatic monocarboxylic acid*. The aliphatic chain can be either *saturated* (no double bonds between carbons) or *unsaturated* (one or more double bonds between carbons). Saturated

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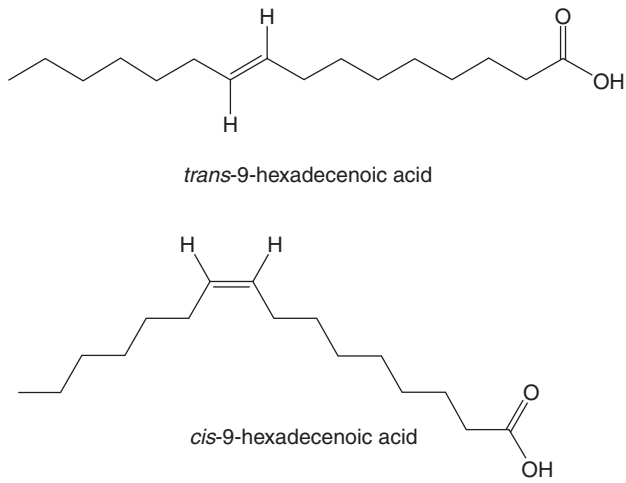


Figure 2.17 *Cis* and *trans* double bond configurations in a fatty acid chain.

fatty acids have the general chemical structure $CH_3(CH_2)_{n-2}CO_2H$, and commonly contain an even number of carbon atoms from $n = 4$ to $n = 20$. In unsaturated fatty acids the double bonds can adopt either a *cis*- or a *trans*-configuration as illustrated in Fig. 2.17. Unsaturated acids can contain one (*monounsaturated*) or several (*polyunsaturated*) double bonds.

There are several conventions for the naming of fatty acids; they can be referred to by their *systematic name* or by a *trivial name*, and it is important to be familiar with both names. A shorthand notation for fatty acids is often employed, which takes the form of a *lipid number*, C:D, where C is the number of carbon atoms and D is the number of double bonds in the fatty acid. This notation can be too general for unsaturated fatty acids since the position of the double bond is not specified; therefore, the lipid number is usually paired with a *delta-n* notation such that each double bond is indicated by Δ^n, where the double bond is located on the n^{th} carbon–carbon bond, counting from the carboxylic acid end. Each double bond is preceded by a *cis*- or *trans*- prefix, indicating the configuration of the bond. Table 2.4 summarizes names and notations for some of the most common fatty acids in foods.

2.4.1.2 Triglycerides

While fatty acids are the most common structural component of lipids, oils and fats are largely composed of mixtures of *triglycerides*. Triglycerides are also known as *triacylglycerols* and are esters of three fatty acids with glycerol. A typical triglyceride structure is depicted in Fig. 2.18. The three fatty acid residues may or may not be the same, i.e. they can be simple or mixed triglycerides. Natural oils and fats will therefore contain a characteristic profile of different fatty acids dependent on their source. For example, fish oils are rich in long-chain polyunsaturated fatty acids (PUFAs) with up to six double bonds, while many vegetable oils are rich in oleic and linoleic acids. Vegetable oils are important in the diet since the body is unable to synthesize linoleic acid, which is an important precursor of *prostaglandins*, a class of hormones that are involved in inflammation and smooth muscle contraction.

Table 2.4 Fatty acid nomenclature.

Systematic name	Trivial name	Lipid number
Butanoic acid	Butyric acid	4:0
Hexanoic acid	Caproic acid	6:0
Octanoic acid	Caprylic acid	8:0
Decanoic acid	Capric acid	10:0
Dodecanoic acid	Lauric acid	12:0
Tetradecanoic acid	Myristic acid	14:0
Hexadecanoic acid	Palmitic acid	16:0
cis-9-Hexadecenoic acid	Palmitoleic acid	16:1, *cis*-Δ^9
Octadecanoic acid	Stearic acid	18:0
cis-9-Octadecenoic acid	Oleic acid	18:1, *cis*-Δ^9
cis, cis-9,12-Octadecadienoic acid	Linoleic acid	18:2, *cis,cis*-$\Delta^{9,12}$
all-*cis*-9,12,15-Octadecatrienoic acid	Linolenic acid	18:3, *cis,cis,cis*-$\Delta^{9,12,15}$
Eicosanoic acid	Arachidic acid	20:0
all-*cis*-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4, *cis,cis,cis,cis*-$\Delta^{5,8,11,14}$
cis-13-Docosenoic acid	Erucic acid	22:1, *cis*-Δ^{13}

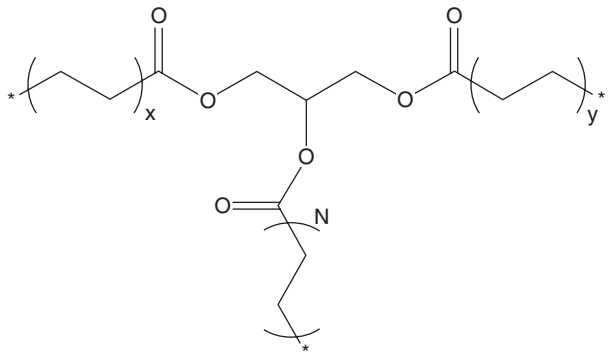


Figure 2.18 The general structure of a triglyceride.

2.4.2 Polymorphism

Polymorphism is an important property of triglyceride crystallization that influences the melting properties of triglycerides. Polymorphic forms have the same chemical composition, but differ in their crystalline structure. Each has a characteristic melting point and there are three basic polymorphic forms: α, β, and β′. The most stable form is β and the least stable is α, and these can be present together within the same sample of a fat. Crystals of β′ fats tend to be small and needle-like, and therefore form better emulsions than the other polymorphic forms.

It is possible to transform a fat from one polymorphic form to another through melting and recrystallization. If a triglyceride is melted and then cooled rapidly it will adopt the α-form. Slow heating will then give a liquid fat that recrystallizes into the β′-form. Repeating this second step yields the stable β-form.

2.4.3 Oil and fat processing

2.4.3.1 Hydrogenation of lipids

Hydrogenation is an important industrial process to convert liquid oils into semi-solid fats for the production of margarine or shortenings. Hydrogenation also increases oxidative stability since unsaturated fatty acids are converted to saturated fatty acids. Briefly, during hydrogenation oils are exposed to hydrogen gas under conditions of high temperature (150–180°C) and pressure (2–10 atm) in the presence of a nickel catalyst. The main reaction is given in Fig. 2.19 alongside the two possible side reactions that may occur: *isomerization* and *double bond migration*.

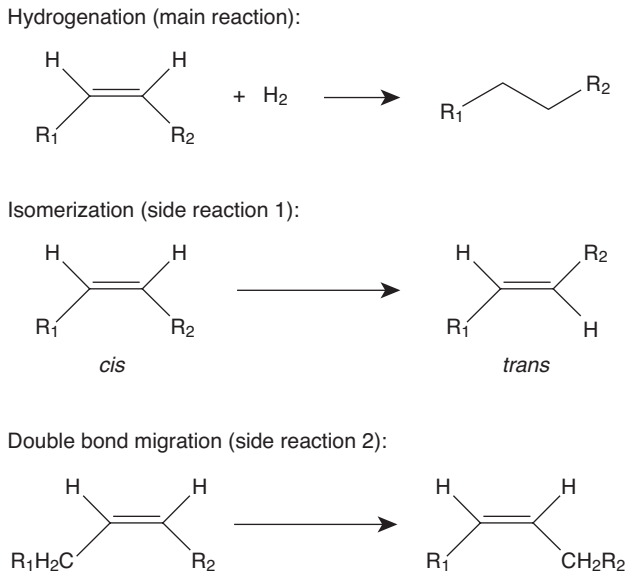


Figure 2.19 Reactions that occur during the hydrogenation of oils and fats.

The isomerization from *cis*- to *trans*-fat is undesirable since the nutritional quality of the fat is reduced. Indeed, the consumption of *trans*-fat arising from partial hydrogenation is linked to an increased risk of coronary heart disease. There has consequently been a worldwide drive to eliminate the consumption of *trans*-fat in the diet. Double bond migration is also linked to a reduction of nutritional quality. Given the occurrence of side reactions, it is important to optimize the *selectivity* of hydrogenation by use of optimal catalysts and conditions.

2.4.3.2 Interesterification

Naturally occurring fats do not contain a random distribution of fatty acids amongst their triglycerides. This is important since the physical characteristics of fats are affected by the fatty acid distribution in triglycerides as well as the overall nature of the fatty acids present. To improve the physical consistency of fats it is possible to perform *interesterification*, which rearranges the fatty acids so that they become distributed randomly among the triglyceride molecules.

Interesterification can be achieved through heating fats at high temperature (< 200°C) for long periods, but it is more efficient to employ catalysts that speed up the process (30 min) and lower the temperature required (50°C). Sodium methoxide is the most

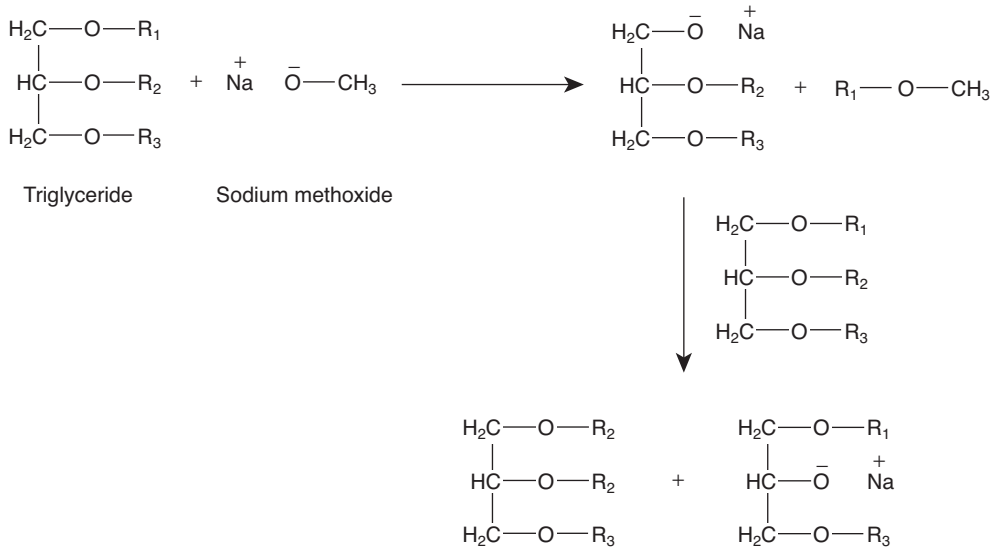


Figure 2.20 Sodium methoxide catalyzed interesterification of triglycerides.

popular catalyst for this process which is shown in Fig. 2.20.

In addition to chemically catalyzed interesterification, it is possible to use enzymes to catalyze a similar process known as *transesterification*. In this case fungal lipases are used to modify palm oils that are rich in 1,3-dipalmityl, 2-oleyl glycerol (POP triglycerides) to yield a fat with an identical profile of POP, POS (1-palmityl, 2-oleyl, 3-stearyl glycerol) and SOS (1,3-distearyl, 2-oleyl glycerol) triglycerides to cocoa butter, which is more expensive (hence value is added).

2.4.4 Lipid oxidation

2.4.4.1 Mechanism

Lipid oxidation is a major cause of food spoilage and causes the generation of off-flavours and off-odours that are termed *rancid*. The fundamental mechanism of lipid oxidation is that of *autoxidation*, which comprises three steps of *initiation*, *propagation* and *termination* as shown in Fig. 2.21. The initiation step involves the generation of highly reactive *free radicals* (molecules having unpaired electrons). These then react with atmospheric oxygen to generate *peroxy radicals* (ROO·), and a chain reaction is set in motion until terminated by the formation of non-radical products.

Autoxidation is accelerated at higher temperature and is more rapid for fats containing polyunsaturated fatty acids. Autoxidation is also sensitive to small concentrations of *antioxidants* or *pro-oxidants*. Pro-oxidants are predominantly metal ions (e.g. iron) that increase the rate of lipid oxidation, either through free radical initiation, acceleration of hydroperoxide (ROOH) decomposition, or activation of molecular oxygen (O_2) to give reactive singlet oxygen ($^1O_2$) and peroxy radicals.

2.4.5 Antioxidants

Antioxidants are substances that can retard the autoxidation of lipids. Both natural and synthetic antioxidants are available as permitted food additives,

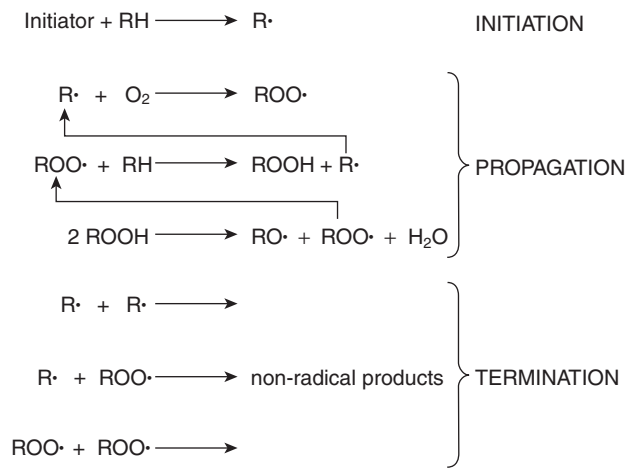
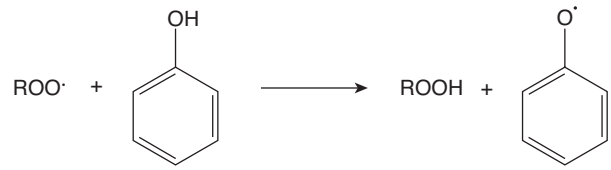
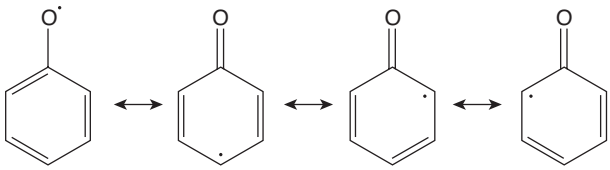


Figure 2.21 The reactions occurring during the autoxidation of lipids.



Free radical intermediate scavenged by antioxidant



Stabilization of antioxidant free radical by resonance hybridization

Figure 2.22 Free radical scavenging by a phenol ring.

and these are mainly phenolic compounds bearing various ring substitutions. The mechanisms of antioxidant activity are a topic of active research, particularly for naturally occurring polyphenols that are abundant in many plant-derived foods. In the case of most synthetic antioxidants, the antioxidant acts as a *radical scavenger* to block the propagation step of autoxidation (see Fig. 2.22).

2.5 Minor components of foods

2.5.1 Permitted additives

The naturally occurring components of foods have a wide range of functional properties that contribute to overall product quality. However, in some cases we may wish to use an additive to enhance food quality, be it for better appearance, texture, flavour, nutritive value or shelf-life. The use of such food additives, especially those that are synthetic in origin, is strictly regulated by government legislation. Briefly summarized here are some of the colours and preservatives that are used as food additives.

2.5.1.1 Colours

Colour is a key sensory component of food and therefore many processed foods contain added colorants. These colorants can be naturally occurring pigments or synthetic dyes, but in recent years there have been moves away from the use of synthetic dyes due to consumer demands.

There are several naturally occurring pigments that are used in foods which are derived from plants, insects and bacteria, including chlorophylls, carotenoids and anthocyanins. *Chlorophylls* are green pigments present in leafy vegetables, fruit, algae and photosynthetic bacteria. Chlorophylls are unstable to heat and insoluble in water; therefore, chlorophyll derivatives (e.g. copper chlorophyllin) are used as added colorants.

Carotenoid pigments give yellow and orange colours in fruits and vegetables, and can be subdivided into carotenes (hydrocarbons) and xanthophylls (contain oxygen). β-Carotene gives the orange colour to carrots, while lycopene occurs in tomatoes and astaxanthin is the molecule responsible for the pink colour of salmon. Carotenoids are of interest for other than their colour since they are generally found to possess antioxidant properties.

Anthocyanins are polyphenolic compounds found in flowers, fruit and vegetables, and give rise to red, violet and blue colours. Fruits such as blackcurrants, blackberries, blueberries, raspberries, strawberries and grapes are particularly rich in anthocyanins. Anthocyanins are the glycoside form of anthocyanidins and, like carotenoids, are noted for their antioxidant properties. Anthocyanins find widespread use as food colours in confectionery and soft drinks, but are of limited use in some foods as their colour is not stable outside of the acid pH range.

Synthetic dyes or artificial food colorants are commonly *azo dyes* (e.g. carmoisine, amaranth), whose colour originates from the azo group (R_1-N=N-R_2). The R-groups in azo dyes are normally aromatic systems, giving a conjugated double bond system that allows for a range of colours (yellow, orange, red, brown). Other synthetic dyes are triarylmethanes (green S, brilliant blue FCF), xanthenes (erythrosine) and quinolines (quinoline yellow).

2.5.1.2 Preservatives

Preservatives are added to food either to prevent rancidity or to prevent microbial growth. Antioxidants have been discussed earlier and can be naturally occurring or synthetic in origin; synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate. Antimicrobial agents are used to prevent spoilage by bacteria, yeast and moulds, and include sulphur dioxide (in the form of SO_2 generating sulphites), benzoic acid (or benzoates), sorbic acid

Vitamin C (Ascorbic Acid)

Ascorbic acid is required for the formation of intercellular substances in the body including dentine, cartilage, and the protein network of bone. Hence, it is important in tooth formation, the healing of broken bones, and the healing of wounds. It may be important to oxidation–reduction reactions in the body and to the production of certain hormones. Vitamin C, like vitamin E, enhances the absorption of iron.

There are several claims that have been attributed to vitamin C such as prevention of colds and removal of cholesterol from the blood as demonstrated in a study conducted on rats. The significance of the rat study for humans is yet to be determined and the cold prevention claim is not supported by the medical profession or the FDA. It has been shown that exceedingly high doses of the vitamin taken for extended periods of time can cause some problems. Infants born to mothers who have been on high doses of the vitamin for a long time have been born with a dependence on the higher doses. A deficiency of the vitamin causes scurvy (spongy, bleeding gums, loss of teeth, swollen joints), fragile capillary walls, and impaired healing of wounds. Excellent sources of the vitamin are orange juice, tomato juice, green peppers, broccoli, cabbage, and brussels sprouts. Potatoes are a fair source because we eat large amounts. Many fruits contain fair amounts of vitamin C.

The food technologist must be aware that vitamin C is easily destroyed by oxidation and heat. It also can be lost in cooking water during processing. Steps must be taken to minimize the losses, and fortification may be necessary before or after processing to ensure sufficient nutrient amounts.

MINERALS

When testing for minerals in the food laboratory, the first step is to remove all water and then all organic matter from the product being tested. After these steps, most of the food (often more than 97%) is gone. What remains is the inorganic material or ash. The human body consists of about 3% minerals, most of which is in the skeletal system. Although the minerals exist is minute amounts, their functions are necessary for normal growth and reproduction. Minerals are generally categorized as "major" and "trace" based on the amounts in the body. Here, a very brief overview of the importance of each mineral is discussed. Further reading on the subject is recommended for students of nutrition. All minerals are important but only two are included on the nutritional labels; thus these are mentioned first.

Iron

Iron is considered a trace mineral because it is needed in such small amounts. Of all the required nutrients, iron may be consumed in insufficient amounts in the industrialized world more commonly than any other. One reason may be the poor absorption of iron sources such as the iron phosphates and iron phytates found in plants. Animal sources and those from soluble salts used in food fortification are generally absorbed more efficiently. Iron is an essential part of both the blood pigment hemoglobin and the muscle pigment myoglobin. It is included in some enzymes. Deficiencies of iron cause anemia. The amount of iron needed is related to growth rate

and blood loss. Women who are of menstruating age should take special caution to ensure sufficient intake. Iron toxicities are rare but care should be taken when storing iron supplement tablets. As few as 6 to 12 tablets could prove fatal if taken by a small child. Good sources of iron are liver, meats, eggs, oatmeal, and wheat flour. As mentioned previously, both vitamin E and vitamin C have been shown to aid in iron absorption.

Calcium

Calcium is one of the minerals that humans require in the greatest amounts (phosphorus is the other). It is required for bone and tooth structure and is necessary for the function of nerves and muscles. Calcium is also needed in the blood clotting mechanism. Deficiencies can lead to osteoporosis, especially in older women. It is suggested that increased calcium intake, especially when young, may help prevent this in later years. Vitamin D is essential for calcium absorption and lactose has been shown to aid in this also. This makes vitamin D fortified milk an excellent, if not the best source of calcium. Calcium deficiencies may be widespread in our society, but unlike most other deficiencies, symptoms are not apparent until later in life. Care should be taken to ensure sufficient calcium intake.

Other Major Minerals

Sodium

In humans, sodium is required in the extracellular fluids to maintain osmotic equilibrium and body-fluid volume. As salt is a major food ingredient there is little evidence of deficiencies except in diseases involving prolonged vomiting or diarrhea. The consumption of salt and other sodium sources should be constrained. Sodium has been shown to aggravate hypertension (high blood pressure) and it is recommended that daily consumption levels be kept between 1100 and 3300 mg. The average in the typical American diet is closer to 6000 mg.

Chlorine

Chlorine is also involved in extracellular fluids as the major negatively charged ion. It has a role in controlling blood pH and is necessary in the production of hydrochloric acid of gastric juice. It is a component of table salt and is never really lacking in the diet. If great losses of body fluids occur, as in vigorous exercise, the amount of chlorine may have to be replenished.

Potassium

Potassium is present in the body cells as the chief intracellular cation and is associated with the function of muscles and nerves and with the metabolism of carbohydrates. It is important in maintaining the fluid volume inside cells, and the acid–base balance. Good sources of potassium are meats, eggs, oranges, bananas, and fresh milk. Cell membranes are quite permeable to potassium, but as it leaks out, a highly active membrane pump returns it to the cell in exchange for sodium. This is critical because

if as little as 6% of the potassium contained in the cells were to escape into the blood, the heart would stop.

Phosphorus

About 85% of the phosphorus in the body is found combined with calcium. It is also part of the body's major buffers (phosphoric acid and its salts) and it is part of both DNA and RNA, the genetic code material present in every cell. Some lipids contain phosphorus in their structure and these phospholipids help transport other lipids in the blood. Phosphorus also plays a key role in energy transfer as it is part of the energy carrier of the cells, ATP. Some sources of phosphorus are meats, fish, eggs, and nuts.

Magnesium

Magnesium is a minor component of bones and is present in soft tissue cells, where it is involved in protein synthesis. Deficiencies of magnesium are unusual and good food sources are most vegetables, cereals and cereal flours, beans, and nuts.

Sulfur

Sulfur is present in virtually all proteins and plays a most important role in forming the cystein bridges that are essential for protein conformation. Deficiencies of sulfur are associated with protein deficiency and if foods containing sufficient protein are eaten then sulfur amounts will be adequate.

Trace Minerals

Iodine

Iodine is part of the hormone thyroxine which regulates metabolic levels. Deficiency of iodine leads to low-level metabolism, lethargy, and goiter, which results in an enlarged thyroid gland. There is rarely a deficiency of iodine when saltwater fish are available and eaten. Today, iodized salt prevents the deficiency but there is some concern about overconsumption of the mineral. Fast food operations use iodized salt liberally, and iodates are used by some bakeries as dough conditioners. Symptoms of toxic levels are similar to those resulting from a deficiency, that of an enlarged thyroid gland. With the identification of the problem, food industries are reducing use of iodine-containing products but care must still be taken.

Flourine

The flourine ion is present in body tissue in trace amounts and helps to prevent tooth decay. Drinking water is the chief source of flourine and fish is also a good source. If flourine is taken in excessively high doses for long periods of time, it is toxic. Too much flourine in the form of supplements can cause flourosis, a mottling of the tooth enamel.

Copper

Copper aids in the utilization of iron in hemoglobin synthesis and is required by some body enzyme systems. It can be toxic in high concentrations and these can be reached if copper utensils are used for storage or distribution of acid foods (copper tubing in machines that dispense lemonade or other acid beverages). Deficiencies are virtually unknown and some food sources are fruits, beans, peas, eggs, liver, fish, and oysters.

Cobalt

Cobalt is a component of vitamin B_{12}, the only component present in the body known to contain this element. Sufficient amounts of cobalt are present in most foods and some may be absorbed into food from cooking utensils. Even though it is part of vitamin B_{12}, it does not replace the need for the vitamin.

Zinc

Zinc is a cofactor in more than 70 enzymes that perform specific tasks in the eyes, liver, kidneys, muscles, skin, bones, and male reproductive organs. These include carbohydrate and protein metabolism and nucleic acid synthesis. Deficiencies are rare but dwarfism, gonadal atrophy, and possible damage to the immune system have been attributed to a deficiency of the mineral. Good sources are protein-rich foods such as shellfish, meat, and liver.

Manganese

Manganese is needed for normal bone structure, fat production, reproduction, and functioning of the central nervous system. Deficiencies result in bone disorders, sexual sterility, and abnormal lipid metabolism. Meats are a source of the mineral but adequate supplies are found in most human diets.

Selenium

Selenium acts as an antioxidant in conjunction with vitamin E. Its major food sources are meat, seafood, and grains. A deficiency results in anemia, muscle pain, and sometimes heart failure.

Vanadium

Vanadium deficiencies in humans are not known but animal and bird studies have shown growth retardation, deficient lipid metabolism, impairment of reproductive function, and bone growth retardation.

Silicon

Silicon is found in unpolished rice and grains. Certain diseases involving connective tissue are believed to result when it is not present in adequate amounts.

Tin

Tin, occurring naturally in many tissues, is necessary for growth in rats. It is believed essential to the structure of proteins and possibly other biological components. As it is present in many foods, a deficiency has not been noted.

Chromium

Chromium plays a physiological role thought to be related to glucose metabolism, perhaps by enhancing the effectiveness of insulin. Although it is a normal body component, its content decreases with age. Depleted tissue concentrations in humans have been linked to adult-onset diabetes. It has been shown to remedy impaired carbohydrate metabolism in several groups of older people in the United States. Whole, unprocessed foods are the best sources whereas refined foods have less of the mineral.

Aluminum, Boron, and Cadmium

Aluminum, boron, and cadmium are also found in trace amounts in the human body, but neither their roles nor the effects of deficient or excessive amounts are known. Although the affected areas of the brain of those afflicted with Alzheimer's disease have been found to contain excessive amounts of aluminum, the effect of this abnormality on the disease or vice versa is not yet clear. It is recommended, however, to avoid long-term storage of acid foods in aluminum pans.

NATURAL TOXICANTS

Some plants are able to produce compounds that serve as protectants or help ensure reproduction. These compounds may attract pollinating insects or repel animals or insects that may eat the plant. Some of these metabolites are quite toxic to man. Some mushrooms, for example, produce specific nitrogen-containing bases or alkaloids that cause severe physiological effects. Other plants, such as potatoes, may produce alkaloid

Table 2.7. Some Natural Toxicants and Their Food Sources

Toxin	Food Source
Cyanide-generating compounds	Lima beans
Safrole	Spices
Prussic acid	Almonds
Oxalic acid	Spinach, rhubarb
Enzyme inhibitors and hemagglutinins	Soybeans
Gossypol	Cottonseed oil
Goitrogens (interfere with iodine binding by the thyroid gland)	Cabbage
Tyramine	Cheese
Avidin (antagonistic to the growth factor biotin)	Egg white
Thiaminase (destroys vitamin B_1)	Fish and shellfish
Vitamins A and D and methionine (exhibit toxic effects in excessive concentrations)	many foods

Phospholipids, because of their polarity (+ and – charges) at one end of the molecule and nonpolarity at the other, are very good emulsifiers and are used in the food industry in such products as chocolate, salad dressings, and mayonnaise (as a component of egg yolks) to help hold polar and nonpolar components together. In the body, they are an important constituent of cell membranes and are involved in the building of such.

Waxes are not made from glycerol, but from fatty acids and monohydric alcohols of 24 to 36 carbons in length. An ester linkage is needed to combine the two molecules. Waxes serve in the body as protective, water repellent coatings on tissue surfaces. Their function is to prevent overevaporation of moisture or invasion of the tissue with water from the environment. In the food industry, waxes are used in some packaging and as ingredients in some confections and candies for texture or appearance. They are not digestible.

Sphingomyelin is a sphingolipid and is an important constituent of nerves and brain tissue. In this lipid, glycerol is replaced by a long-chained nitrogen-containing alcohol.

There are a number of sterols that have important functions in the body. These are very complex chemical compounds containing an alcohol group to which fatty acids can be esterified. The sterol cholesterol is involved in the composition of bile salts, which play a role in the emulsification of fats in the intestine, hence, in the digestion of fats. Ergosterol, another sterol, may be converted to vitamin D in the body under the influence of sunlight or ultraviolet light.

More information on fats and oils, how they are used in industry, and their oxidation is found in Chapters 8, 9, and 22.

VITAMINS

There are a number of vitamins required in small amounts by the human body for sustaining life and good health. Some are fat-soluble; others are water-soluble. The recommended dietary allowances (RDA) for the vitamins and minerals described here are shown in Table 2.1.

Fat-Soluble Vitamins

Vitamin A

Vitamin A is found only in animals, although a number of plants contain carotene, from which vitamin A can be produced in the body once the plants containing carotene are eaten. Vitamin A may be formed in the body from the yellow pigments (containing carotene) of many fruits and vegetables, especially carrots. Vitamin A, which is required for vision and resistance to infection, is also found in fats and oils, especially in the liver oils of many saltwater fish. Epithelial cells (those cells present in the lining of body cavities and in the skin and glands) also require vitamin A. Deficiency in vitamin A may cause impairment in bone formation, impairment of night vision, malfunction of epithelial tissues, and defects in the enamel of the teeth.

Vitamin A previously was expressed in international units (IU). Because of the different biological activities of vitamin A sources such as beta-carotene, other carotenes, and preformed vitamin A (retinol), vitamin A is now expressed in terms of retinol equivalents (RE). To convert, 1 IU = 0.3 RE. The definition of RE is given by the Food and Nutrition Board as:

1 RE = microgram all-*trans*retinol = 6 micrograms of
beta-carotene = 12 micrograms of other provitamin A carotenoids.

The Reference Daily Intakes (RDIs), on which the % daily values on the nutritional labels are based, for vitamin A are 5000 IU based on the 1968 RDAs. This corresponds to 1500 REs. Vitamin A is routinely added to milk. The 1989 revised RDA for vitamin A is 1000 REs for adult males and 800 for females (see Table 2.1). Nutritional labels are discussed further in Chapter 7.

Vitamin D

Vitamin D (calciferol or activated ergosterol) is necessary for normal tooth and bone formation. Deficiencies in vitamin D result in rickets (deformities of bone, such as bow-legs and curvature of the spine) and tooth defects. Fish oils, and especially fish liver oils, are excellent sources of vitamin D. The human body is also able to make vitamin D by converting sterols such as cholesterol with ultraviolet light from the sun or an artificial source. The RDA for vitamin D was in the past expressed in IUs and 400 was the RDS. It is now expressed in micrograms (μg) as cholecalciferol and 10 μg = 400 IU. Vitamin D, as vitamin A is also routinely added to milk.

Vitamin E

Vitamin E has four different forms (the tocopherols) that have the same name except with the prefixes alpha-, beta-, gamma-, and delta-. The four compounds are closely related, with slight differences in structure, but alpha-tocopherol is the most common and serves as an antioxidant that serves to prevent the oxidation of body components, such as unsaturated fatty acids. Vitamin E has also been shown to enhance the absorption of iron. Diets excessive in polyunsaturated fats can lead to the formation of peroxidized fatty acids that could reach dangerous levels. Vitamin E may prevent this. Good sources of Vitamin E are corn oil, cottonseed oil, and peanut oil.

While the symptoms of vitamin E deficiency in humans are not clearly established, experiments with various animals have shown that vitamin E deficiency has an adverse effect on reproduction, with apparent irreversible injury to the germinal epithelium. Other symptoms noted in animal studies include injury to the central nervous system, growth retardation, muscular dystrophy, and interference with normal heart action.

The RDA for vitamin E is now given in milligrams of alpha-tocopherol equivalents (α-TE). The old measurement was in IUs and 30 was the RDA (10 α-TE = 30 IU).

Vitamin K

Vitamin K is essential for the synthesis of prothrombin, a compound involved in the clotting of blood. Cabbage, spinach, cauliflower, and liver are especially good sources and it is also synthesized by bacteria in the human intestine. Antibiotic therapy that destroys intestinal organisms can produce deficiencies of vitamin K.

The significant symptom of vitamin K deficiency in humans and in animals is the loss of the ability of the blood to clot which is, of course, a dangerous condition that can result in death whenever bleeding occurs. It is believed that humans ordinarily

receive adequate amounts of vitamin K in the diet. The RDA ranges from 5 μg in infants to 80 μg in males over 25 years of age.

Water-Soluble Vitamins

Vitamin B_1 (Thiamin)

Thiamin is involved in all bodily oxidations that lead to the formation of carbon dioxide. It is also necessary for nerve function, appetite, and normal digestion as well as growth, fertility, and lactation. The symptoms of thiamin deficiency are retardation of growth, palpitation and enlargement of the heart, hypertension, and beri-beri. The various effects of a disturbance of the nerve centers such as forgetfulness or difficulty in thinking are other manifestations of vitamin B_1 deficiency.

Important to the food scientist is the sensitivity of vitamin B_1 to sulfur dioxide (SO_2) and to sulfite salts. SO_2 destroys vitamin B_1 and should not be used as a preservative in foods that are a major source of the vitamin. FDA and meat inspection laws prohibit such use. Thiamin is stable to heat in acid foods but is less so in neutral and alkaline foods. This should be taken into account when processing foods.

Good sources of the vitamin are fresh pork, wheat germ, and cereals containing bran and some fair sources are beef and lamb.

Vitamin B_2 (Riboflavin)

Vitamin B_2 makes up a part of enzyme systems involved in the oxidation and reduction of different materials in the body. Deficiency of riboflavin generally results in growth retardation and may result in vision impairment, scaling of the skin, and lesions on mucous tissues. Neuritis is another effect of deficiency. Milk, liver, and eggs are good sources while meats and leafy green vegetables are moderate sources. Riboflavin is sensitive to light, so packaging such as cardboard and other light-resistant containers for milk are used.

Niacin (Nicotinic Acid)

Niacin is part of enzyme systems regulating reduction reactions in the body. It is also a compound that dilates blood vessels. It is part of the coenzyme nicotinamide adenine dinucleotide (NAD) which is involved in glucose breakdown. Deficiency of niacin causes pellagra (a disease that causes diarrhea, dermatitis, nervous disorders, and sometimes death). Good sources are yeast, meat, fish, poultry, peanuts, legumes, and whole grain.

Vitamin B_6 (Pyridoxine)

Other substances closely related to this vitamin are pyridoxal and pyridoxamine. Vitamin B_6 is part of the enzyme system that removes CO_2 from the acid group (COOH) of certain amino acids and transfers amine groups (NH_2) from one compound to another in the body. Although the vitamin is needed for processes such as those mentioned, it seems a deficiency does not cause a well-recognized disease. Bananas, barley, grain cereals with bran, muscle meat, liver, and green vegetables are all among sources of

this vitamin. Vitamin B_6 has been used by women taking steroid contraceptive pills, and it has been used in treatment of such ailments as premenstrual syndrome (PMS) but this is not recommended without valid blood or urine tests to show low B_6 levels.

Biotin

Biotin is reported to be a coenzyme in the synthesis of aspartic acid, which plays a part in a deaminase system and in other processes involving the fixation of carbon dioxide. Deficiency of this compound is unusual, although it can be tied up by a substance in raw egg whites called aviden and the deficiency has been demonstrated in feeding studies with mice. Because of the production of biotin by microbial flora of the intestines, the dietary requirement for this compound is unknown. Liver is an excellent source of biotin and peanuts, peas, beans, and whole cooked eggs are good sources.

Pantothenic Acid

Pantothenic acid, a vitamin required for normal growth, nerve development, and normal skin, is a component of coenzyme A and others involved in metabolism. Pantothenic acid is widespread in foods, so obvious symptoms of a deficiency are rare in humans. Organs of animals (liver, heart, kidney), eggs, whole wheat products, and peanuts are excellent sources.

Choline

Choline is generally listed with the B vitamins and is consumed in adequate amounts in a normal diet as well as being produced by intestinal microbial flora (other growth factors such as biotin, para-aminobenzoic acid, and inositol are also produced in this manner). It is a component of cell membranes and brain tissue and it functions as part of the substance acetylcholine, which is one of the brain's principal neurotransmitters.

Vitamin B_{12} (Cyanocobalamin)

This vitamin is a very complex chemical compound and is the largest vitamin molecule. It is required for the normal development of red blood cells, and a deficiency in vitamin B_{12} causes acute pernicious anemia and a variety of other disorders. Cobalt is part of Vitamin B_{12}'s structure, thus giving rise to the requirement for this mineral in nutrition. Some vitamin B_{12} is synthesized by bacteria in the intestine but intake of 2 μg per day for adults is recommended.

Folacin

Folacin is required for the formation of blood cells by the bone marrow and is involved in the formation of the blood pigment hemoglobin. It is also required for the synthesis of some amino acids. Deficiency results in some types of anemia including pernicious anemia. The RDA is increased from 180 μg to 400 μg for pregnant women, as it is believed the vitamin may act in prevention of some birth defects. It is part of the coenzyme system tetrahydrofolate (THF), which is required for the synthesis of new genetic material and therefore new cells. Liver, leafy vegetables, legumes, cereal grains, and nuts are all sources of the vitamin.

Vitamin C (Ascorbic Acid)

Ascorbic acid is required for the formation of intercellular substances in the body including dentine, cartilage, and the protein network of bone. Hence, it is important in tooth formation, the healing of broken bones, and the healing of wounds. It may be important to oxidation–reduction reactions in the body and to the production of certain hormones. Vitamin C, like vitamin E, enhances the absorption of iron.

There are several claims that have been attributed to vitamin C such as prevention of colds and removal of cholesterol from the blood as demonstrated in a study conducted on rats. The significance of the rat study for humans is yet to be determined and the cold prevention claim is not supported by the medical profession or the FDA. It has been shown that exceedingly high doses of the vitamin taken for extended periods of time can cause some problems. Infants born to mothers who have been on high doses of the vitamin for a long time have been born with a dependence on the higher doses. A deficiency of the vitamin causes scurvy (spongy, bleeding gums, loss of teeth, swollen joints), fragile capillary walls, and impaired healing of wounds. Excellent sources of the vitamin are orange juice, tomato juice, green peppers, broccoli, cabbage, and brussels sprouts. Potatoes are a fair source because we eat large amounts. Many fruits contain fair amounts of vitamin C.

The food technologist must be aware that vitamin C is easily destroyed by oxidation and heat. It also can be lost in cooking water during processing. Steps must be taken to minimize the losses, and fortification may be necessary before or after processing to ensure sufficient nutrient amounts.

MINERALS

When testing for minerals in the food laboratory, the first step is to remove all water and then all organic matter from the product being tested. After these steps, most of the food (often more than 97%) is gone. What remains is the inorganic material or ash. The human body consists of about 3% minerals, most of which is in the skeletal system. Although the minerals exist is minute amounts, their functions are necessary for normal growth and reproduction. Minerals are generally categorized as "major" and "trace" based on the amounts in the body. Here, a very brief overview of the importance of each mineral is discussed. Further reading on the subject is recommended for students of nutrition. All minerals are important but only two are included on the nutritional labels; thus these are mentioned first.

Iron

Iron is considered a trace mineral because it is needed in such small amounts. Of all the required nutrients, iron may be consumed in insufficient amounts in the industrialized world more commonly than any other. One reason may be the poor absorption of iron sources such as the iron phosphates and iron phytates found in plants. Animal sources and those from soluble salts used in food fortification are generally absorbed more efficiently. Iron is an essential part of both the blood pigment hemoglobin and the muscle pigment myoglobin. It is included in some enzymes. Deficiencies of iron cause anemia. The amount of iron needed is related to growth rate

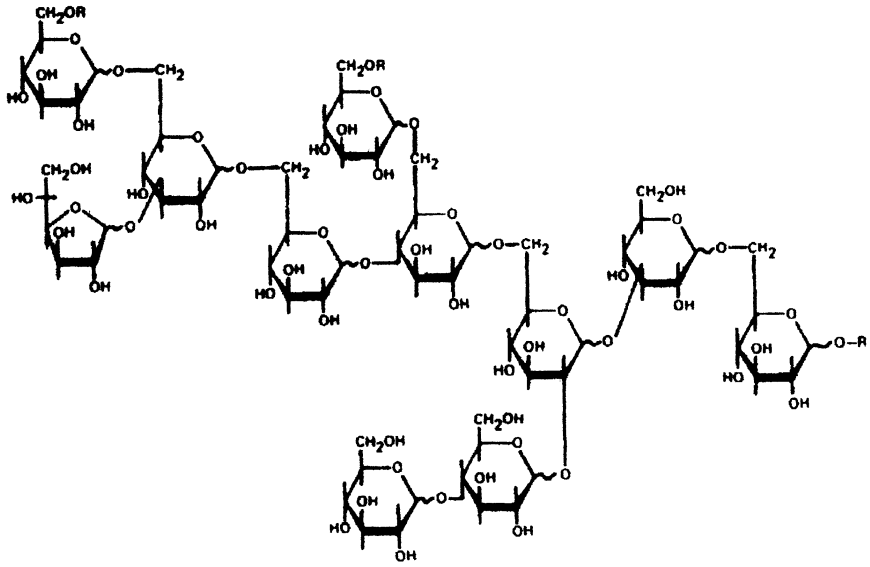


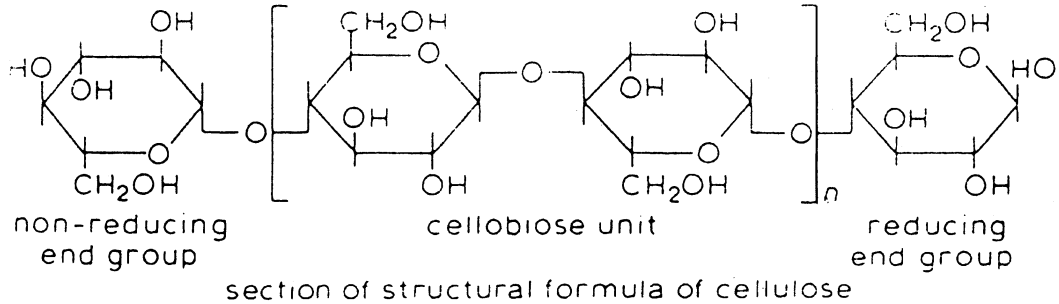
Figure 2.7. Polydextrose chemical bonding.

citric acid (Fig. 2.7). It is highly branched and, because of its unusual structure, is not readily digested and contains only one calorie per gram. It can be used as a "bulking agent" in low-calorie products to replace sugar, bind water, and add to textural attributes without greatly increasing calories.

Fibers

Dietary fiber includes the nondigestible carbohydrates. These may be either water-soluble or water-insoluble. Both have nutritional significance. The water-insoluble group, which includes wheat products and wheat bran, is believed to reduce chances of colon cancer by increasing bulk and diluting the effect of secondary bile acids. The water-soluble fibers, such as some cereal brans and pectin, are believed to lower serum cholesterol levels by binding with bile acids and causing removal of cholesterol in the feces. Short discussions of cellulose, pectin, and gums are given here.

Cellulose comprises most of the structural material in plants and is the main component of many industrially important substances including wood, paper, and fibers such as cotton. Chemically, cellulose is a linear polymer of D-glucose molecules with beta-1,4 bonds:

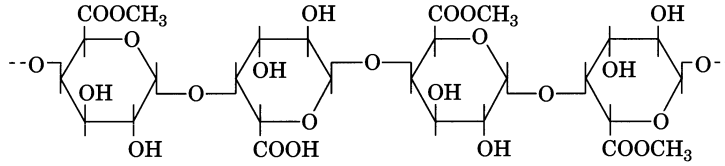


section of structural formula of cellulose

In nature, cellulose occurs in fibers that contain very high mechanical strength and are insoluble in water. The cellulose molecule is elongated and rigid. The hydroxyl group protruding from the chain may readily form hydrogen bonds, giving cellulose this tough texture. Upon hydrolysis, cellobiose and finally glucose are produced. Beta-glucosidase is an effective cellulase enzyme and is able to catalyze cleavage of the alpha-1,4 linkages. These enzymes occur in fungi and in some bacteria. Man and most animals are unable to digest cellulose because they lack cellulases in their digestive tracts. Ruminants such as cows and sheep are able to utilize cellulose as energy sources because of the presence of cellulase-excreting microorganisms in the rumen. Similarly, termites utilize microorganisms living in their digestive tracts also to hydrolyze wood cellulose.

Treating cellulose with dilute acid yields "microcrystalline cellulose" which consists of very fine particles that can be used as an indigestible noncaloric food additive. Many low-calorie muffins and other baked products use this type of ingredient to increase volume and mass without adding calories.

Pectin is a water-soluble fiber that exists in the intercellular spaces, the middle lamella, of plant tissues. Pectins are heteropolysaccharides, as their structure includes more than one type of molecule. The unbranched chains consist of polygalacturonic acid molecules joined with a 1,4 linkage. The galacturonic acid units may be esterified (an acid is combined with an alcohol) with methyl alcohol:



This structure shows 75% esterification of methylation.

Upon acid, alkali, or enzyme hydrolysis, the methoxy groups are first removed, leaving an unesterified chain of galacturonic acid called pectic acid. Further hydrolysis results in individual galacturonic acid units.

Depending on the degree of esterification and the chain length, pectins vary in their jelly-forming properties. With normal degrees of esterification of about 50% to 70%, pectins will form gels with addition of acid and sugar and will do so rather slowly as the jelly cools. Increasing the esterification to above 70% will cause the gel to form quicker and this "rapid set" pectin is chosen for products such as jams and preserves, where a gel is needed before the particles of fruit sink. Another variation is the low-methoxy pectin, which has less methoxylation (less than 50%) and will form a gel with less sugar and the addition of a divalent ion such as calcium (Ca^{a+}). This pectin is used in the low-calorie fruit spreads.

Gums, because of their ability to give highly viscous solutions at relatively low concentrations, are chosen as gelling, stabilizing, and suspending agents. Gums all have hydrophilic properties and are therefore included in the water-soluble fibers. They can be obtained from several sources including exudate gums (gum arabic), bean gums (locust bean gum), seed gums (guar gum), and seaweed gums (agar, algin, and carrageenan). They are used in many foods such as candies and other confections, fruit sauces, syrups, toppings, spreads, baked goods, salad dressings, and beverages.

All are heteropolysaccharides and their sources and composition are outlined in Table 2.4.

Table 2.4. Some Common Gums

Gum	Source	Components
Gum arabic	Acadia trees	L-Arabinose, L-rhamnose, D-galactose, D-glucuronic acid
Locust bean gum	Carob bean	D-galacto-D-mannoglycan, pentoglycan, protein, cellulose, ash
Guar gum	Guar plant seed	D-galacto-D-mannoglycan chain with single galactose branches
Agar	Algae (*Rhodophyceae*)	Agarose, agaropectin
Algin	Giant kelp (*Macrocystis pyrifera*)	Anhydro-1, 4-beta-D-Mannuronic acid, L-glucuronic acid
Carrageenan	Irish moss (*Chondrus crispus*)	—[a]

[a]Three fractions have been isolated and all consist of salts or sulfate esters with a ratio of sulfate to hexose units of close to unity.

LIPIDS

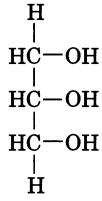
The term lipid is generally defined as the heterogeneous group of substances, associated with living systems, which have the common property of insolubility in water but solubility in nonpolar solvents such as hydrocarbons or alcohols. The major members of this group are the fats and oils, which are given the most emphasis in this section. Other members of the lipid group include waxes, phospholipids, sphingolipids, and sterols which all have important roles in food biochemistry and nutrition and will be mentioned briefly. Nutritionally, the fats and oils contain 9 calories per gram while proteins and carbohydrates contain only 4 calories per gram.

Fats and Oils

When an alcohol and an acid are joined producing a water molecule, the result is an ester.

$$R\text{–}OH + R_1\text{–}COOH \rightarrow R\text{–}00CR_1$$
$$\text{alcohol} + \text{acid} \rightarrow \text{ester}$$

Glycerol (glycerine) is a polyhydric alcohol that is capable of forming three esters with different acid components.



glycerol

6

chapter

Moisture and Total Solids Analysis

Robert L. Bradley, Jr.

6.1 INTRODUCTION

Moisture assays can be one of the most important analyses performed on a food product and yet one of the most difficult from which to obtain accurate and precise data. This chapter describes various methods for moisture analysis—their principles, procedures, applications, cautions, advantages, and disadvantages. Water activity measurement also is described, since it parallels the measurement of total moisture as an important stability and quality factor. With an understanding of techniques described, one can apply appropriate moisture analyses to a wide variety of food products.

6.1.1 Importance of Moisture Assay

One of the most fundamental and important analytical procedures that can be performed on a food product is an assay for the amount of moisture (1–3). The dry matter that remains after moisture removal is commonly referred to as **total solids**. This analytical value is of great economic importance to a food manufacturer because water is an inexpensive filler. The following listing gives some examples in which moisture content is important to the food processor.

1. Moisture is a quality factor in the preservation of some products and affects stability in:
   a. dehydrated vegetables and fruits
   b. dried milks
   c. powdered eggs
   d. dehydrated potatoes
   e. spices and herbs.
2. Moisture is used as a quality factor for:
   a. jams and jellies, to prevent sugar crystallization
   b. sugar syrups
   c. prepared cereals—conventional, 4–8%; puffed, 7–8%.
3. Reduced moisture is used for convenience in packaging or shipping of:
   a. concentrated milks
   b. liquid cane sugar (67% solids) and liquid corn sweetener (80% solids)
   c. dehydrated products (these are difficult to package if too high in moisture)
   d. concentrated fruit juices.
4. Moisture (or solids) content is often specified in compositional standards (i.e., Standards of Identity):
   a. Cheddar cheese must be ≤39% moisture.
   b. Enriched flour must be ≤15% moisture.
   c. Pineapple juice must have soluble solids of ≥10.5 °Brix (conditions specified).
   d. Glucose syrup must have ≥70% total solids.
   e. The percentage of added water in processed meats is commonly specified.
5. Computations of the nutritional value of foods require that you know the moisture content.
6. Moisture data are used to express results of other analytical determinations on a uniform basis (i.e., dry weight basis).

6.1.2 Moisture Content of Foods

The moisture content of foods varies greatly, as shown in Table 6-1 (4). Water is a major constituent of most food products. The approximate, expected moisture content of a food can affect the choice of the method of measurement. It also can guide the analyst in determining the practical level of accuracy required when measuring moisture content, relative to other food constituents.

6.1.3 Forms of Water in Foods

The ease of water removal from foods depends on how it exists in the food product. The three states of water in food products are:

1. **Free water**—This water retains its physical properties and thus acts as the dispersing agent for colloids and the solvent for salts.
2. **Adsorbed water**—This water is held tightly or is occluded in cell walls or protoplasm and is held tightly to proteins.
3. **Water of hydration**—This water is bound chemically, for example, lactose monohydrate; also some salts such as $Na_2SO_4 \cdot 10H_2O$.

Depending on the form of the water present in a food, the method used for determining moisture may measure more or less of the moisture present. This is the reason for official methods with stated procedures (5–7). However, several official methods may exist for a particular product. For example, the AOAC International methods for cheese include: Method 926.08, vacuum oven; 948.12, forced draft oven; 977.11, microwave oven; 969.19, distillation (5). Usually, the first method listed by AOAC International is preferred over others in any section.

6.1.4 Sample Collection and Handling

General procedures for sampling, sample handling and storage, and sample preparation are given in Chapter 5. These procedures are perhaps the greatest potential source of error in any analysis. Precautions must be taken to minimize inadvertent **moisture losses or gains** that occur during these steps. Obviously, any exposure of a sample to the open atmosphere should be as short as possible. Any heating of a sample by friction during grinding should be minimized. Headspace in the

6-1 table **Moisture Content of Selected Foods**

Food Item	*Approximate Percent Moisture (Wet Weight Basis)*
Cereals, bread, and pasta	
Wheat flour, whole-grain	10.3
White bread, enriched (wheat flour)	13.4
Corn flakes cereal	1.7–3.5
Crackers saltines	4.1
Macaroni, dry, enriched	10.2
Dairy products	
Milk, whole, fluid, 3.3% fat	88.0
Yogurt, plain, low fat	85.1
Cottage cheese, low fat or 2% milk fat	79.3
Cheddar cheese	36.8
Ice cream, vanilla	61.0
Fats and oils	
Margarine, regular, hard, corn, hydrogenated	15.7
Butter, with salt	15.9
Oil—soybean, salad, or cooking	0
Fruits and vegetables	
Watermelon, raw	91.5
Oranges, raw, California navels	86.8
Apples, raw, with skin	83.9
Grapes, American type, raw	81.3
Raisins	15.4
Cucumbers, with peel, raw	96.0
Potatoes, microwaved, cooked in skin, flesh and skin	72.0
Snap beans, green, raw	90.3
Meat, poultry, and fish	
Beef, ground, extra lean, raw	63.2
Chicken, broilers and fryers, light meat, meat and skin, raw	68.6
Finfish, flatfish (flounder and sole species), raw	79.1
Egg, whole, raw, fresh	75.3
Nuts	
Walnuts, black, dried	4.4
Peanuts, all types, dry roasted with salt	1.6
Peanut butter, smooth style, with salt	1.2
Sweeteners	
Sugar, granulated	0
Sugar, brown	1.6
Honey, strained or extracted	17.1

From USDA Nutrient Database for Standard Reference, with modification. Release 14 (July 2001). http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl

sample storage container should be minimal because moisture is lost from the sample to equilibrate the container environment against the sample. It is critical to control temperature fluctuations since moisture will migrate in a sample to the colder part. To control this potential error, remove the entire sample from the container, reblend quickly, then remove a test portion (8 and 9).

To illustrate the need for optimum efficiency and speed in weighing samples for analysis, Bradley and Vanderwarn (10) showed, using shredded Cheddar cheese (2–3 g in a 5.5-cm aluminum foil pan), that moisture loss within an analytical balance was a straight line function. The rate of loss was related to the relative humidity. At 50% relative humidity, it required only 5 sec to lose 0.01% moisture. This time doubled at 70% humidity, or 0.01% moisture loss in 10 sec. While one might expect a curvilinear loss, the moisture loss was actually linear over a 5-min study interval. These data demonstrate the necessity of absolute control during collection of samples through weighing, before drying.

6.2 OVEN DRYING METHODS

In **oven drying methods**, the sample is heated under specified conditions, and the loss of weight is used to calculate the moisture content of the sample. The amount of moisture determined is highly dependent on the type of oven used, conditions within the oven, and the time and temperature of drying. Various oven methods are approved by AOAC International for determining the amount of moisture in many food products. The methods are simple, and many ovens allow for simultaneous analysis of large numbers of samples. The time required may be from a few minutes to over 24 hr.

6.2.1 General Information

6.2.1.1 Removal of Moisture

Any oven method used to evaporate moisture has as its foundation the fact that the boiling point of water is 100°C; however, this considers only pure water at sea level. Free water is the easiest of the three forms of water to remove. However, if 1 g molecular weight (1 mol) of a solute is dissolved in 1.0 L of water, the boiling point would be raised by 0.512°C. This boiling point elevation continues throughout the moisture removal process as more and more concentration occurs.

Moisture removal is sometimes best achieved in a two-stage process. Liquid products (e.g., juices, milk) are commonly predried over a **steam bath** before drying in an oven. Products such as bread and field dried grain are often air dried, then ground and oven dried, with the moisture content calculated from moisture loss at both air and oven drying steps. Particle size, particle size distribution, sample sizes, and surface area during

drying influence the rate and efficiency of moisture removal.

6.2.1.2 Decomposition of Other Food Constituents

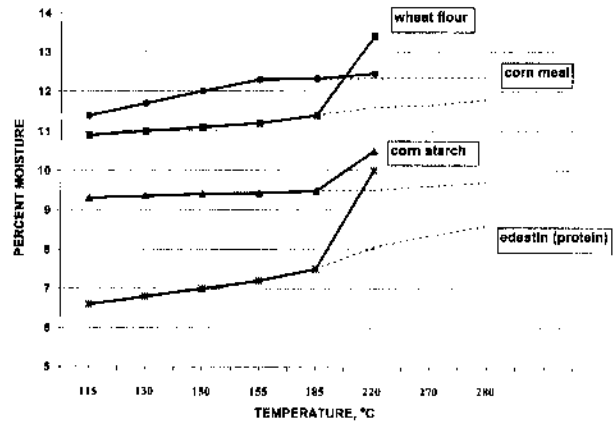
Moisture loss from a sample during analysis is a function of time and temperature. Decomposition enters the picture when time is extended too much or temperature is too high. Thus, most methods for food moisture analysis involve a compromise between time and a particular temperature at which limited decomposition might be a factor. One major problem exists in that the physical process must separate all the moisture without decomposing any of the constituents that could release water. For example, carbohydrates decompose at 100°C according to the following reaction:

$$C_6H_{12}O_6 \rightarrow 6C + 6H_2O \qquad [1]$$

The moisture generated in carbohydrate decomposition is not the moisture that we want to measure. Certain other chemical reactions (e.g., sucrose hydrolysis) can result in utilization of moisture, which would reduce the moisture for measurement. A less serious problem, but one that would be a consistent error, is the loss of **volatile constituents**, such as acetic, propionic, and butyric acids; and alcohols, esters, and aldehydes among flavor compounds. While weight changes in oven drying methods are assumed to be due to moisture loss, weight gains also can occur due to oxidation of unsaturated fatty acids and certain other compounds.

Nelson and Hulett (11) determined that moisture was retained in biological products to at least 365°C, which is coincidentally the critical temperature for water. Their data indicate that among the decomposition products at elevated temperatures were CO, CO_2, CH_4, and H_2O. These were not given off at any one particular temperature but at all temperatures and at different rates at the respective temperature in question.

By plotting moisture liberated against temperature, curves were obtained that show the amount of moisture liberated at each temperature (Fig. 6-1). Distinct breaks were shown that indicated the temperature at which decomposition became measurable. None of these curves showed any break before 184°C. Generally, proteins decompose at temperatures somewhat lower than required for starches and celluloses. Extrapolation of the flat portion of each curve to 250°C gave a true moisture content based on the assumption that there was no adsorbed water present at the temperature in question.



6-1 figure Moisture content of several foods held at various temperatures in an oven. The hyphenated line extrapolates data to 250°F, the true moisture content. [Reprinted with permission from (11), pp. 40–45. Copyright 1920, American Chemical Society.]

6.2.1.3 Temperature Control

Drying methods utilize specified drying temperatures and times, which must be carefully controlled. Moreover, there may be considerable variability of temperature, depending on the type of oven used for moisture analysis. One should determine the extent of variation within an oven before relying on data collected from its use.

Consider the temperature variation in three types of ovens: **convection (atmospheric)**, **forced draft**, and **vacuum**. The greatest temperature variation exists in a convection oven. This is because hot air slowly circulates without the aid of a fan. Air movement is obstructed further by pans placed in the oven. When the oven door is closed, the rate of temperature recovery is generally slow. This is dependent also upon the load placed in the oven and upon the ambient temperature. A 10°C temperature differential across a convection oven is not unusual. This must be considered in view of anticipated analytical accuracy and precision. A convection oven should not be used when precise and accurate measurements are needed.

Forced draft ovens have the least temperature differential across the interior of all ovens, usually not greater than 1°C. Air is circulated by a fan that forces air movement throughout the oven cavity. Forced draft ovens with air distribution manifolds appear to have added benefit where air movement is horizontal across shelving. Thus, no matter whether the oven shelves are filled completely with moisture pans or only half filled, the result would be the same for a particular sample. This has been demonstrated using a Lab-Line oven (Melrose Park, IL) in which three stacking configurations for the pans were used (10). In one configuration, the oven shelves were filled with as many pans holding

2–3 g of Cheddar cheese as the forced draft oven could hold. In the two others, one half of the full load of pans with cheese was used with the pans (1) in orderly vertical rows with the width of one pan between rows, or (2) staggered such that pans on every other shelf were in vertical alignment. The results after drying showed no difference in the mean value or the standard deviation.

Two features of some **vacuum ovens** contribute to a wider temperature spread across the oven. One feature is a glass panel in the door. Although from an educational point of view it may be fascinating to observe some samples in the drying mode, the glass is a heat sink. The second feature is the way by which air is bled into the oven. If the air inlet and discharge are on opposite sides, conduct of air is virtually straight across the oven. Some newer models have air inlet and discharge manifolds mounted top and bottom. Air movement in this style of vacuum oven is upward from the front, then backward to the discharge in a broad sweep. The effect is to minimize cold spots as well as to exhaust moisture in the interior air.

6.2.1.4 Types of Pans for Oven Drying Methods

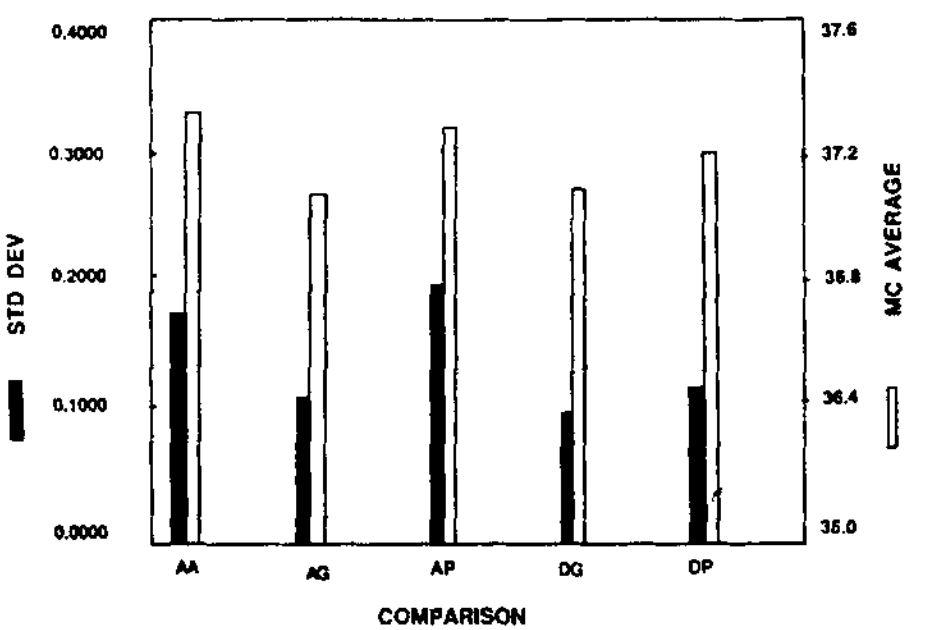
Pans used for moisture determinations are varied in shape and may or may not have a cover. The AOAC International (5) moisture pan is about 5.5 cm in diameter with an insert cover. Other pans have covers that slip over the outside edge of the pan. These pans, while reusable, are expensive, in terms of labor costs to clean appropriately to allow reuse.

Pan covers are necessary to control loss of sample by spattering during the heating process. If the cover is metal, it must be slipped to one side during drying to allow for moisture evaporation. However, this slipping of the cover also creates an area where spattering will result in product loss. Examine the interior of most moisture ovens and you will detect odor and deposits of burned-on residue, which, although undetected at the time of occurrence, produce erroneous results and large standard deviations (10).

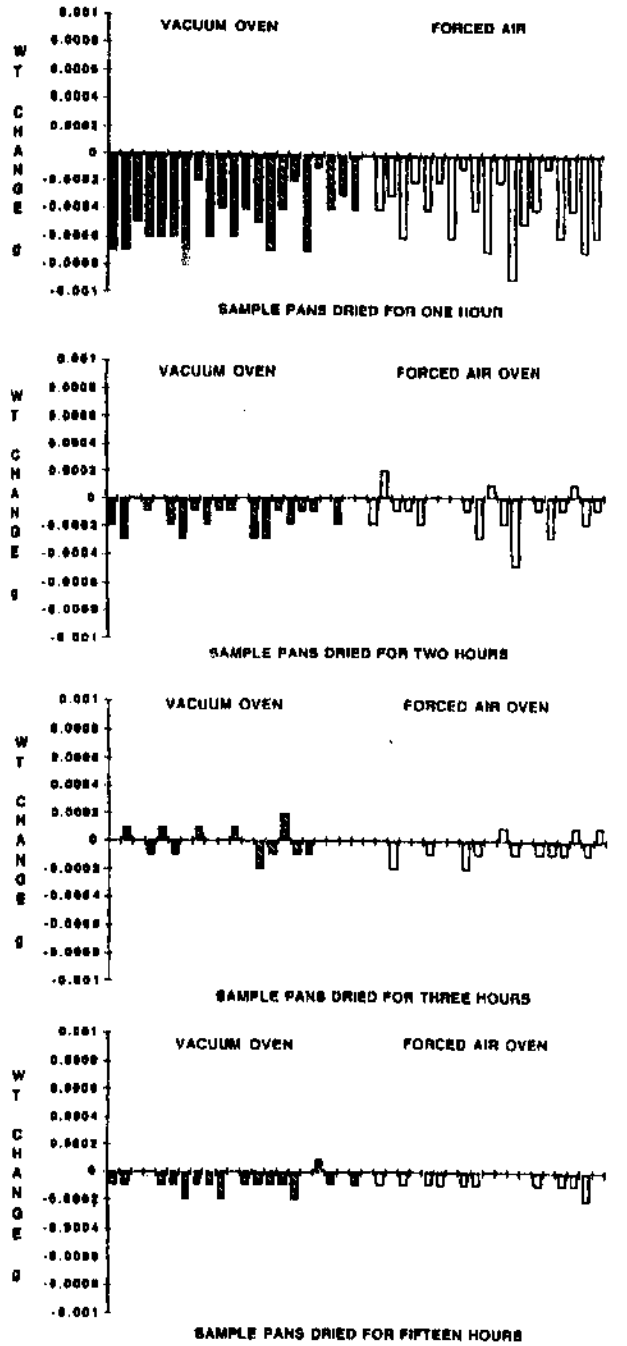
Consider the use of **disposable pans** whenever possible; then purchase **glass fiber discs** for covers. At 5.5 cm in diameter, these covers fit perfectly inside disposable aluminum foil pans and prevent spattering while allowing the surface to breathe. Paper filter discs foul with fat and thus do not breathe effectively. Consider the evidence presented in Fig. 6-2, derived from at least 10 replicate analyses of the same cheese with various pans and covers. These data prove two points: (1) fat does spatter from pans with slipped covers and (2) fiberglass is the most satisfactory cover.

6.2.1.5 Handling and Preparation of Pans

The handling and preparation of pans before use requires consideration. Use only **tongs** to handle any pan. Even fingerprints have weight. All pans must be oven treated to prepare them for use. This is a factor of



6-2 figure Effect of various pan and cover combinations on the moisture content (MC) of Cheddar cheese. Standard deviations show precision of the analysis. Pans: A = AOAC, D = disposable; Covers: A = AOAC, G = glass fiber disc, P = filter paper disc. [Reprinted from (10), *The Journal of AOAC INTERNATIONAL*, 2001, Vol. 84, pp. 570–592. Copyright, 2001, by AOAC INTERNATIONAL.]



6-3 figure Effect of drying new disposable aluminum moisture pans in a vacuum and forced draft oven at 100°C. The sensitivity of the balance was 0.0001 g. [Reprinted from (10), *The Journal of AOAC INTERNATIONAL*, 2001, Vol. 84, pp. 570–592. Copyright, 2001, by AOAC INTERNATIONAL.]

major importance unless disproved by the technologist doing moisture determinations with a particular type of pan. To illustrate the weight change that occurs with disposable aluminum pans, consider the examples in Fig. 6-3. Disposable aluminum pans must be vacuum oven dried for 3 hr before use. At 3 hr and 15 hr in either a vacuum or forced draft oven at 100°C, pans varied in their weight within the error of the balance, or 0.0001 g (10). Store dry moisture pans in a functioning **desiccator**. The glass fiber covers should be dried for one hour before use.

6.2.1.6 Control of Surface Crust Formation (Sand Pan Technique)

Some food materials tend to form a semipermeable crust or lump together during drying, which will contribute to erratic and erroneous results. To control this problem, analysts use the **sand pan technique**. Clean, dry sand and a short glass stirring rod are preweighed into a moisture pan. Subsequently, after weighing in a sample, the sand and sample are admixed with the stirring rod left in the pan. The remainder of the procedure follows a standardized method if available; otherwise the sample is dried to constant weight. The purpose of the sand is twofold: to prevent **surface crust** from forming and to disperse the sample so evaporation of moisture is less impeded. The amount of sand used is a function of sample size. Consider 20–30 g sand/3 g sample to obtain desired distribution in the pan. Similar to the procedure, applications, and advantages of using sand, other heat-stable inert materials such as diatomaceous earth can be used in moisture determinations, especially for sticky fruits.

The inert matrices such as sand and **diatomaceous earth** function to disperse the food constituents and minimize the retention of moisture in the food products. However, the analyst must ascertain that the inert matrix used does not give erroneous results for the assay because of decomposition or entrapped moisture loss. Test the sand or other inert matrix for weight loss before using in any method. Add approximately 25 g of sand into a moisture pan and heat at 100°C for 2 hr and weigh to 0.1 mg. Add 5 ml water and mix with the matrix using a glass rod. Heat dish, matrix, cover, and glass rod for at least 4 hr at 100°C, reweigh. The difference between weighing must be less than 0.5 mg for any suitable matrix (12).

6.2.1.7 Calculations

Moisture and total solids contents of foods can be calculated as follows using oven drying procedures:

$$\%\ \text{Moisture (wt/wt)} = \frac{\text{wt } H_2O \text{ in sample}}{\text{wt of wet sample}} \times 100 \quad [2]$$

$$\%\ \text{Moisture (wt/wt)} = \frac{\text{wt of wet sample} - \text{wt of dry sample}}{\text{wt of wet sample}} \times 100 \quad [3]$$

$$\% \text{ Total solids (wt/wt)} = \frac{\text{wt of dry sample}}{\text{wt of wet sampe}} \times 100 \quad [4]$$

6.2.2 Forced Draft Oven

When using a forced draft oven, the sample is rapidly weighed into a predried moisture pan covered and placed in the oven for an arbitrarily selected time if no standardized method exists. Drying time periods for this method are 0.75–24 hr (Table 6-2), depending on the food sample and its pretreatment; some liquid samples are dried initially on a steam bath at 100°C to minimize spattering. In these cases, drying times are shortened to 0.75–3 hr. A forced draft oven is used with or without a steam table predrying treatment to determine the solids content of fluid milks (AOAC Method 990.19, 990.20).

An alternative to selecting a time period for drying is to weigh and reweigh the dried sample and pan until two successive weighings taken 30 min apart agree within a specified limit, for example, 0.1–0.2 mg for a 5-g sample. The user of this second method must be aware of sample transformation, such as browning which suggests moisture loss of the wrong form. Lipid oxidation and a resulting sample weight gain can occur at high temperatures in a forced draft oven. Samples high in carbohydrates should not be dried in a forced draft oven but rather in a vacuum oven at a temperature no higher than 70°C.

6.2.3 Vacuum Oven

By drying under reduced pressure (25–100 mmHg), one is able to obtain a more complete removal of water and volatiles without decomposition within a 3–6-hr drying time. Vacuum ovens need a dry air purge in addition to temperature and vacuum controls to operate within method definition. In older methods, a vacuum flask is used, partially filled with concentrated sulfuric acid as the desiccant. One or two air bubbles per second are passed through the acid. Recent changes now stipulate an air trap that is filled with calcium sulfate containing an indicator to show moisture saturation. Between the trap and the vacuum oven is an appropriately sized rotameter to measure air flow (100–120 ml/min) into the oven.

The following are important points in the use of a vacuum drying oven:

1. **Temperature** used depends on the product, such as 70°C for fruits and other high-sugar products. Even with reduced temperature, there can be some decomposition.
2. If the product to be assayed has a high concentration of **volatiles**, you should consider the use of a correction factor to compensate for the loss.
3. Analysts should remember that in a **vacuum**, heat is not conducted well. Thus pans must be placed directly on the metal shelves to conduct heat.
4. **Evaporation** is an endothermic process; thus, a pronounced cooling is observed. Because of the cooling effect of evaporation, when several samples are placed in an oven of this type, you will note that the temperature will drop. Do not attempt to compensate for the cooling effect by increasing the temperature, otherwise samples during the last stages of drying will be overheated.
5. The **drying time** is a function of the total moisture present, nature of the food, surface area per unit weight of sample, whether sand is used as a dispersant, and the relative concentration of sugars and other substances capable of retaining moisture or decomposing. The drying interval is determined experimentally to give reproducible results.

6.2.4 Microwave Analyzer

Determination of moisture in food products has traditionally been done using a standard oven, which, though accurate, can take many hours to dry a sample. Other methods have been developed over the years including infrared and various types of instruments that utilize halogen lamps or ceramic heating elements. They were often used for "spot checking" because of their speed, but they lacked the accuracy of the standard oven method. The introduction of microwave moisture/solids analyzers in the late 1970s gave laboratories the accuracy they needed and the speed they wanted. **Microwave moisture analysis**, often called **microwave drying**, was the first precise and rapid technique that allowed some segments of the food industry to make in-process adjustment of the moisture content in food products before final packaging. For example, processed cheese could be analyzed and the composition adjusted before the blend was dumped from the cooker. The ability to adjust the composition of a product in-process helps food manufacturers reduce production costs, meet regulatory requirements, and ensure product consistency. Such control could effectively pay for the microwave analyzer within a few months.

A particular microwave moisture/solids analyzer (CEM Corporation, Matthews, NC), or equivalent, is specified in the AOAC International procedures for total solids analysis of processed tomato products (AOAC Method 985.26) and moisture analysis of meat and poultry products (AOAC Method 985.14).

The general procedure for use of a microwave moisture/solids analyzer has been to set the microprocessor controller to a percentage of full power to control

6-2 table **Forced Draft Oven Temperature and Times for Selected Foods**

Product	*Dry on Steam Bath*	*Oven Temperature (°C ± 2)*	*Time in Oven (Hr)*
Buttermilk, liquid	X[1]	100	3
Cheese, natural type only		100	16.5 ± 0.5
Chocolate and cocoa		100	3
Cottage cheese		100	3
Cream, liquid and frozen	X	100	3
Egg albumin, liquid	X	130	0.75
Egg albumin, dried	X	100	0.75
Ice cream and frozen desserts	X	100	3.5
Milk	X	100	3
Whole, low fat, and skim		100	3
Condensed skim		100	3
Evaporated			
Nuts: almonds, peanuts, walnuts		130	3

From (6), p. 492, with permission, *Standard Methods for the Examination of Dairy Products*, Robert T. Marshall (Ed.). Copyright 1992 by the American Public Health Association. [1]X = samples must be partially dried on steam bath before being placed in oven.

the microwave output. Power settings are dependent upon the type of sample and the recommendations of the manufacturer of the microwave moisture analyzer. Next, the internal balance is tared with two sample pads on the balance. As rapidly as possible, a sample is placed between the two pads, then pads are centered on the pedestal and weighed against the tare weight. Time for the drying operation is set by the operator and "start" is activated. The microprocessor controls the drying procedure, with percentage moisture indicated in the controller window. Some newer models of microwave moisture analyzers have a temperature control feature to precisely control the drying process, removing the need to guess appropriate time and power settings for specific applications. These new models also have a smaller cavity that allows the microwave energy to be focused directly on the sample.

There are some considerations when using a microwave analyzer for moisture determination: (1) the sample must be of a uniform, appropriate size to provide for complete drying under the conditions specified; (2) the sample must be centrally located and evenly distributed, so some portions are not burned and other areas underprocessed; and, (3) the amount of time used to place an appropriate sample weight between the pads must be minimized to prevent moisture loss or gain before weight determination. Sample pads also should be considered. There are several different types, including fiberglass and quartz fiber pads. For optimum results, the pads should not absorb microwave energy, as this can cause the sample to burn, nor should they fray easily, as this causes them to lose weight and can affect the analysis. In addition, they should absorb liquids well.

Another style of microwave oven that includes a vacuum system is used in some food plants. This vacuum microwave oven will accommodate one sample in triplicate or three different samples at one time. In 10 min, the results are reported to be similar to 5 hr in a vacuum oven at 100°C. The vacuum microwave oven is not nearly as widely used as conventional microwave analyzers, but can be beneficial in some applications.

Microwave drying provides a fast, accurate method to analyze many foods for moisture content. The method is sufficiently accurate for routine assay. The distinct advantage of rapid analysis far outweighs its limitation of testing only single samples (13).

6.2.5 Infrared Drying

Infrared drying involves penetration of heat into the sample being dried, as compared to heat conductivity and convection with conventional ovens. Such heat penetration to evaporate moisture from the sample can significantly shorten the required drying time, to 10–25 min. The infrared lamp used to supply heat to the sample results in a filament temperature of 2000–2500 K. Factors that must be controlled include distance of the infrared source from the dried material and thickness of the sample. The analyst must be careful that the sample does not burn or case harden while drying. Infrared drying ovens may be equipped with forced ventilation to remove moisture air and an analytical balance to read moisture content directly. No infrared drying moisture analysis techniques are approved by AOAC currently. However, because of the speed of analysis, this technique is suited for qualitative in-process use.

6.2.6 Rapid Moisture Analyzer Technology

Many rapid moisture/solids analyzers are available to the food industry. In addition to those based on infrared and microwave drying as described previously, compact instruments that depend on high heat are available, such as analyzers that detect moisture levels from 50 ppm to 100% using sample weights of 150 mg to 40 g (e.g., Computrac, Arizona Instrument Corporation, Phoenix, AZ). Using a digital balance, the test sample is placed on an aluminum pan or filter paper and the heat control program (with a heating range of 25°C to 275°C) elevates the test sample to a constant temperature. As the moisture is driven from the sample, the instrument automatically weighs and calculates the percentage moisture or solids. This technology is utilized to cover a wide range of applications within the food industry and offers quick and accurate results within minutes. These analyzers are utilized for both production and laboratory use with results comparable to reference methods although the method is not currently AOAC approved.

6.3 DISTILLATION PROCEDURES

6.3.1 Overview

Distillation techniques involve codistilling the moisture in a food sample with a high boiling point solvent that is immiscible in water, collecting the mixture that distills off, and then measuring the volume of water. Two distillation procedures are in use today: **direct** and **reflux distillations**, with a variety of solvents. For example, in direct distillation with immiscible solvents of higher boiling point than water, the sample is heated in mineral oil or liquid with a flash point well above the boiling point for water. Other immiscible liquids with boiling point only slightly above water can be used (e.g., toluene, xylene, and benzene). However, reflux distillation with the immiscible solvent toluene is the most widely used method.

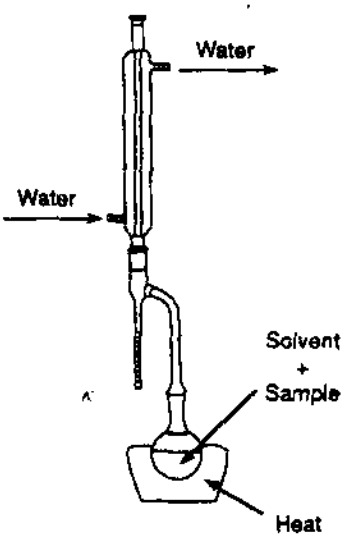
Distillation techniques were originally developed as rapid methods for quality control work, but they are not adaptable to routine testing. The distillation method is an AOAC-approved technique for moisture analysis of spices (AOAC Method 986.21), cheese (AOAC Method 969.19), and animal feeds (AOAC Method 925.04). It also can give good accuracy and precision for nuts, oils, soaps, and waxes.

Distillation methods cause less thermal decomposition of some foods than oven drying at high temperatures. Adverse chemical reactions are not eliminated but can be minimized by using a solvent with a lower boiling point. This, however, will increase distillation times. Water is measured directly in the distillation procedure (rather than by weight loss), but reading the volume of water in a receiving tube may be less accurate than using a weight measurement.

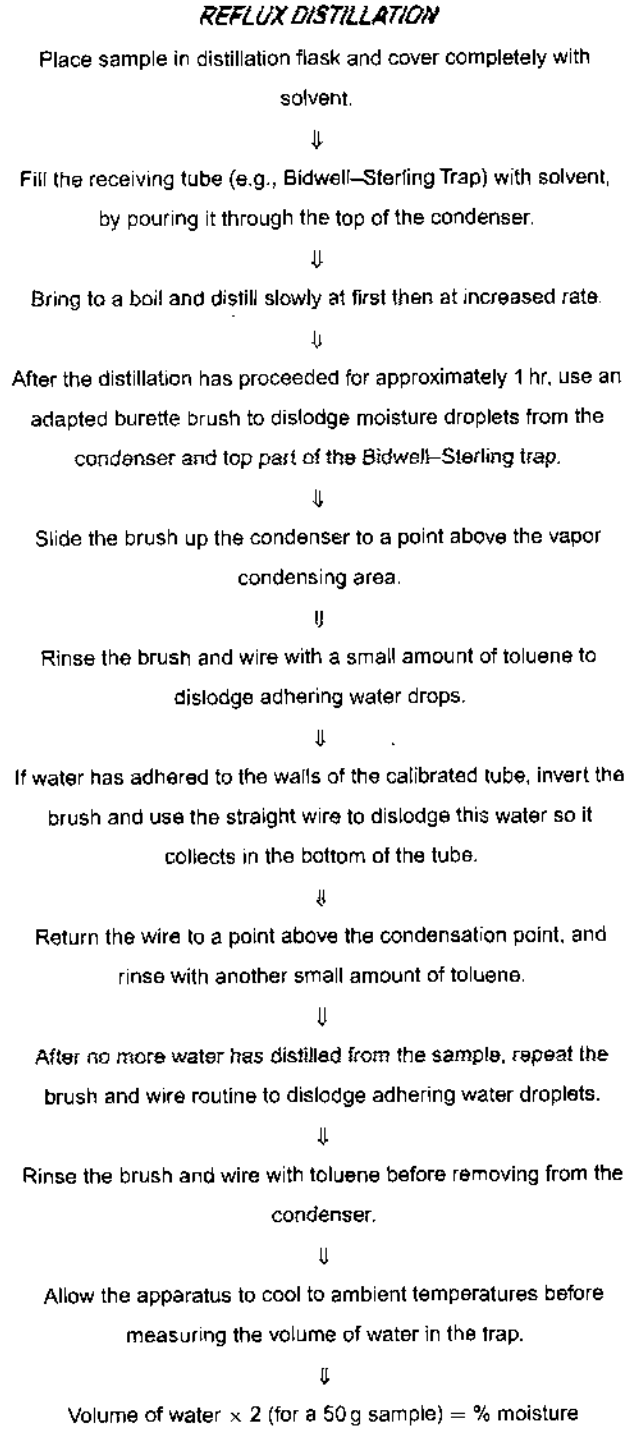
6.3.2 Reflux Distillation with Immiscible Solvent

Reflux distillation uses either a solvent less dense than water (e.g., toluene, with a boiling point of 110.6°C; or xylene, with a boiling range of 137–140°C) or a solvent more dense than water (e.g., tetrachlorethylene, with a boiling point of 121°C). The advantage of using this last solvent is that material to be dried floats; therefore it will not char or burn. In addition, there is no fire hazard with this solvent.

A **Bidwell–Sterling moisture trap** (Fig. 6-4) is commonly used as part of the apparatus for reflux distillation with a solvent less dense than water. The distillation procedure using such a trap is described in Fig. 6-5, with emphasis placed on dislodging adhering water drops, thereby minimizing error. When the toluene in the distillation just starts to boil, the analyst will observe a hazy cloud rising in the distillation flask. This is a vaporous emulsion of water in toluene. Condensation occurs as the vapors rise, heating the vessel, the Bidwell–Sterling trap, and the bottom of the condenser. It also is hazy at the cold surface of the condenser, where water droplets are visible. The emulsion inverts and becomes toluene dispersed in water. This turbidity clears very slowly on cooling.



6-4 figure Apparatus for reflux distillation of moisture from a food. Key to this setup is the Bidwell–Sterling moisture trap. This style can be used only where the solvent is less dense than water.



6-5 figure Procedures for reflux distillation with toluene using a Bidwell–Sterling trap. Steps to dislodge adhering moisture drops are given.

Three potential sources of error with distillation should be eliminated if observed:

1. Formation of emulsions that will not break. Usually this can be controlled by allowing the apparatus to cool after distillation is completed *and before reading the amount of moisture in* the trap.
2. Clinging of water droplets to dirty apparatus. Clean glassware is essential, but water seems to cling even with the best cleaning effort. A burette brush, with the handle end flattened so it will pass down the condenser, is needed to dislodge moisture droplets.
3. Decomposition of the sample with production of water. This is principally due to carbohydrate decomposition to generate water ($C_6H_{12}O_6 \rightarrow 6H_2O + 6C$). If this is a measurable problem, discontinue method use and find an alternative procedure.

6.4 CHEMICAL METHOD: KARL FISCHER TITRATION

The **Karl Fischer titration** is particularly adaptable to food products that show erratic results when heated or submitted to a vacuum. This is the method of choice for determination of water in many low-moisture foods such as dried fruits and vegetables (AOAC Method 967.19 E–G), candies, chocolate (AOAC Method 977.10), roasted coffee, oils and fats (AOAC Method 984.20), or any low-moisture food high in sugar or protein. The method is quite rapid, accurate, and uses no heat. This method is based on the fundamental reaction described by Bunsen in 1853 (14) involving the reduction of iodine by SO_2 in the presence of water:

$$2H_2O + SO_2 + I_2 \rightarrow C_5H_2SO_4 + 2HI \qquad [5]$$

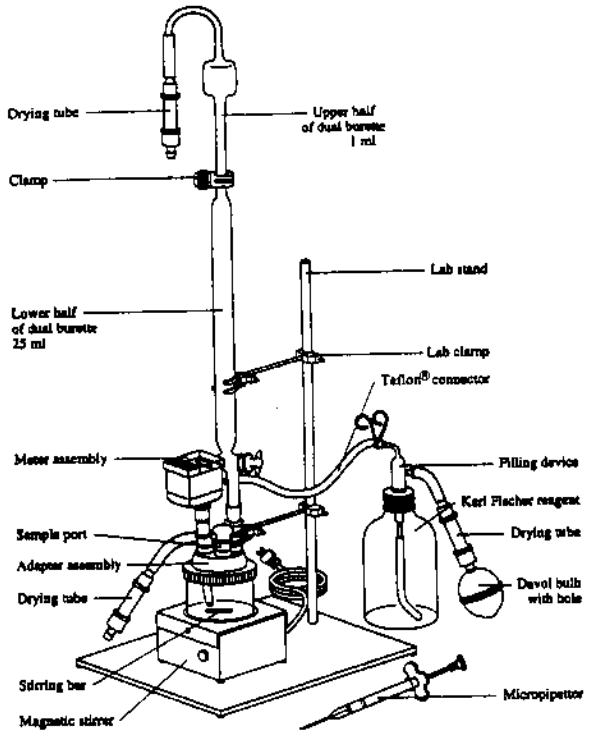
This was modified to include methanol and pyridine in a four-component system to dissolve the iodine and SO_2:

$$C_5H_5N \cdot I_2 + C_5H_5N \cdot SO_2 + C_5H_5N + H_2O \rightarrow 2C_5H_5N \cdot HI + C_5H_5N \cdot SO_3 \qquad [6]$$

$$C_5H_5N \cdot SO_3 + CH_3OH \rightarrow C_5H_5N(H)SO_4 \cdot CH_3 \qquad [7]$$

These reactions show that for each mol of water, 1 mol of iodine, 1 mol of SO_2, 3 mol of pyridine, and 1 mol of methanol are used. For general work, a methanolic solution is used that contains these components in the ratio of 1 iodine: 3 SO_2: 10 pyridine, and at a concentration so that 3.5 mg of water = 1 ml of reagent. A procedure for standardizing this reagent is given below.

In a **volumetric titration** procedure (Fig. 6-6), iodine and SO_2 in the appropriate form are added to the sample in a closed chamber protected from atmospheric moisture. The excess of I_2 that cannot react with the water can be determined visually. The endpoint



6-6 figure Karl Fischer titration unit. (Courtesy of Lab Industries, Inc., Berkeley, CA.)

color is dark red-brown. Some instrumental systems are improved by the inclusion of a potentiometer to electronically determine the endpoint, which increases the sensitivity and accuracy. Instruments are available to automatically perform the Karl Fischer moisture analysis by the **conductometric method**.

The volumetric titration procedure described above is appropriate for samples with a moisture content greater than ~0.03%. A second type of titration, referred to as **coulometric titration**, is ideal for products with very low levels of moisture, from 0.03% down to parts per million (ppm) levels. In this method, iodine is electrolytically generated to titrate the moisture. The amount of iodine required to titrate the moisture is determined by the current needed to generate the iodine.

In a Karl Fischer volumetric titration, the **Karl Fischer reagent** (KFR) is added directly as the titrant if the moisture in the sample is accessible. However, if moisture in a solid sample is inaccessible to the reagent, the moisture is extracted from the food with an appropriate solvent (e.g., methanol). (Particle size affects efficiency of extraction directly.) Then the methanol extract is titrated with KFR.

The obnoxious odor of pyridine makes it an undesirable reagent. Therefore, researchers have experimented with other amines capable of dissolving iodine and sulfur dioxide. Some aliphatic amines and several other heterocyclic compounds were found suitable. On the basis of these new amines, **one-component reagents** (solvent and titrant components together) and **two-component reagents** (solvent and titrant components separate) have been prepared. The one-component reagent may be more convenient to use, but the two-component reagent has greater storage stability.

Before the amount of water found in a food sample can be determined, a **KFR water (moisture) equivalence** (KFReq) must be determined. The KFReq value represents the equivalent amount of moisture that reacts with 1 ml of KFR. Standardization must be checked before each use because the KFReq will change with time.

The KFReq can be established with **pure water**, a **water-in-methanol standard**, or **sodium tartrate dihydrate**. Pure water is a difficult standard to use because of inaccuracy in measuring the small amounts required. The water-in-methanol standard is premixed by the manufacturer and generally contains 1 mg of water/ml of solution. This standard can change over prolonged storage periods by absorbing atmospheric moisture. Sodium tartrate dihydrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) is a primary standard for determining KFReq. This compound is very stable, contains 15.66% water under all conditions expected in the laboratory, and is the material of choice to use.

The KFReq is calculated as follows using sodium tartrate dihydrate:

$$\text{KFReq (mg } H_2O\text{/ml)} = \frac{36 \text{ g } H_2O\text{/mol } Na_2C_4H_4O_6 \cdot 2H_2O \times S \times 1000}{230.08 \text{ g/mol} \times A} \quad [8]$$

where:

KFReq = Karl Fischer reagent moisture equivalence
S = weight of sodium tartrate dihydrate (g)
A = ml of KFR required for titration of sodium tartrate dihydrate

Once the KFReq is known, the moisture content of the sample is determined as follows:

$$\%H_2O = \frac{\text{KFReq} \times Ks}{S} \times 100 \quad [9]$$

where:

KFReq = Karl Fischer reagent water (moisture) equivalence
Ks = ml of KFR used to titrate sample
S = weight of sample (mg)

The major difficulties and sources of error in the Karl Fischer titration methods are:

1. **Incomplete moisture extraction**—For this reason, fineness of grind (i.e., particle size) is

important in preparation of cereal grains and some foods.

2. **Atmospheric moisture**—External air must not be allowed to infiltrate the reaction chamber.
3. **Moisture adhering** to walls of unit—All glassware and utensils must be carefully dried.
4. **Interferences** from certain food constituents—**Ascorbic acid** is oxidized by KFR to dehydroascorbic acid to overestimate moisture content; **carbonyl compounds** react with methanol to form acetals and release water, to overestimate moisture content (this reaction also may result in fading endpoints); **unsaturated fatty acids** will react with iodine, so moisture content will be overestimated.

6.5 PHYSICAL METHODS

6.5.1 Electrical Methods

6.5.1.1 Dielectric Method

Moisture content of certain foods can be determined by measuring the change in **capacitance** or **resistance to an electric current** passed through a sample. These instruments require calibration against samples of known moisture content as determined by standard methods. Sample density or weight/volume relationships and sample temperature are important factors to control in making reliable and repeatable measurements by dielectric methods. These techniques can be very useful for process control measurement applications, where continuous measurement is required. These methods are limited to food systems that contain no more than 30–35% moisture.

The moisture determination in dielectric-type meters is based on the fact that the dielectric constant of water (80.37 at 20°C) is higher than that of most solvents. The **dielectric constant** is measured as an index of **capacitance**. As an example, the dielectric method is used widely for cereal grains. Its use is based on the fact that water has a dielectric constant of 80.37, whereas starches and proteins found in cereals have dielectric constants of 10. By determining this properly on samples in standard metal condensers, dial readings may be obtained and the percentage of moisture determined from a previously constructed standard curve for a particular cereal grain.

6.5.1.2 Conductivity Method

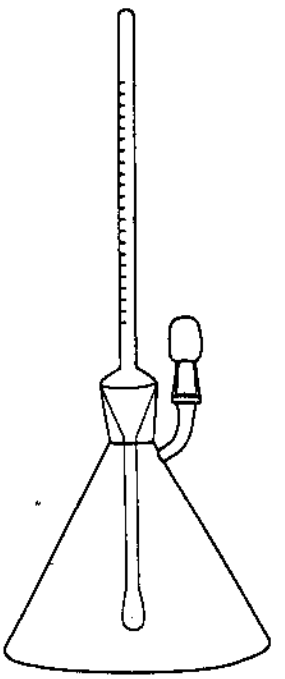
The **conductivity method** functions because the conductivity of an electric current increases with the percentage of moisture in the sample. A modestly accurate and rapid method is created when one measures resistance. Ohm's law states that the strength of an electrical current is equal to the electromotive force divided by the resistance. The electrical resistance of wheat with 13% moisture is seven times as great as that with 14% moisture and 50 times that with 15% moisture. Temperature must be kept constant, and 1 min is necessary for a single determination.

6.5.2 Hydrometry

Hydrometry is the science of measuring **specific gravity** or **density**, which can be done using several different principles and instruments. While hydrometry is considered archaic in some analytical circles, it is still widely used and, with proper technique, is highly accurate. Specific gravity measurements with a **pycnometer**, various types of **hydrometers**, or a **Westphal balance** are commonly used for routine testing of moisture (or solids) content of numerous food products. These include beverages, salt brines, and sugar solutions. Specific gravity measurements are best applied to the analysis of solutions consisting of only one solute in a medium of water.

6.5.2.1 Pycnometer

One approach to measuring specific gravity is a comparison of the weights of equal volumes of a liquid and water in standardized glassware, a **pycnometer** (Fig. 6-7). This will yield density of the liquid compared



6-7 figure Pycnometer.

to water. In some texts and reference books, *20/20* is given after the specific gravity number. This indicates that the temperature of both fluids was 20°C when the weights were measured. Using a clean, dry pycnometer at 20°C, the analyst weighs it empty, fills it to the full point with distilled water at 20°C, inserts the thermometer to seal the fill opening, and then touches off the last drops of water and puts on the cap for the overflow tube. The pycnometer is wiped dry in case of any spillage from filling and is reweighed. The density of the sample is calculated as follows:

$$\frac{\text{weight of sample-filled pycnometer} - \text{weight of empty pycnometer}}{\text{weight of water-filled pycnometer} - \text{weight of empty pycnometer}} = \text{density of sample} \quad [10]$$

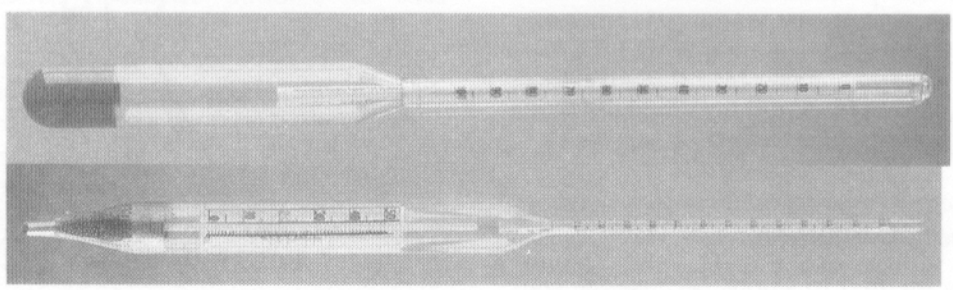
This method is used for determining alcohol content in alcoholic beverages (e.g., distilled liquor, AOAC Method 930.17), solids in sugar syrups (AOAC Method 932.14B), and solids in milk (AOAC Method 925.22).

6.5.2.2 Hydrometer

A second approach to measuring specific gravity is based on **Archimedes' principle**, which states that a solid suspended in a liquid will be buoyed by a force equal to the weight of the liquid displaced. The weight per unit volume of a liquid is determined by measuring the volume displaced by an object of standard weight. A hydrometer is a standard weight on the end of a spindle, and it displaces a weight of liquid equal to its own weight (Fig. 6-8). For example, in a liquid of low density, the hydrometer will sink to a greater depth, whereas in a liquid of high density, the hydrometer will not sink as far. Hydrometers are available in narrow and wide ranges of specific gravity. The spindle of the hydrometer is calibrated to read specific gravity directly at 15.5°C or 20°C. A **hydrometer** is not as accurate as a pycnometer, but the speed with which you can do an analysis is a decisive factor. The accuracy of specific gravity measurements can be improved by using a hydrometer calibrated in the desired range of specific gravities.

The rudimentary but surprisingly accurate hydrometer comes equipped with various modifications depending on the fluid to be measured:

1. The Quevenne and New York Board of Health **lactometer** is used to determine the density of milk. The Quevenne lactometer reads from 15 to 40 lactometer units and corresponds to 1.015 to 1.040 specific gravity. For every degree above 60°F, 0.1 lactometer unit is added to the reading, and 0.1 lactometer unit is subtracted for every degree below 60°F.
2. The **Baumé hydrometer** was used originally to determine the density of salt solutions (originally 10% salt), but it has come into much wider use. From the value obtained in the Baumé scale, you can convert to specific gravity of liquids heavier than water. For example, it is used to determine the specific gravity of milk being condensed in a vacuum pan.
3. The **Brix hydrometer** is a type of **saccarometer** used for sugar solutions such as fruit juices and syrups, and one usually reads directly the percentage of sucrose at 20°C. **Balling saccharometers** are graduated to indicate percentage of sugar by weight at 60°F. The terms **Brix** and **Balling** are interpreted as the weight percentage of pure sucrose.
4. **Alcoholometers** are used to estimate the alcohol content of beverages. Such hydrometers are calibrated in 0.1°C or 0.2°C proof to determine the percentage of alcohol in distilled liquors (AOAC Method 957.03).
5. The **Twaddell hydrometer** is only for liquids heavier than water.



6-8 figure Hydrometers. (Courtesy of Cole-Parmer Instrument Company, Vernon Hills, IL.)

6.5.2.3 Westphal Balance

The **Westphal balance** functions on Archimedes' principle such that the plummet on the balance will be buoyed by the weight of liquid equal to the volume displaced. This is more accurate than a hydrometer but less accurate than a pycnometer. It provides measurements to four decimal places. The balance has a plummet that displaces exactly 5 g of water at 15.5°C. If the specific gravity is 1, as would be the case with water at 15.5°C, a gravity weight hung at the 10 mark would bring this device into balance.

The specific gravity measurement of solid objects is made as described below, with the determination of frozen pea maturity given as the example:

1. Weigh peas in air.
2. Immerse peas in solvent.
3. Obtain weight in this solvent.

$$\text{Specific gravity} = \frac{\text{weight in air} \times \text{specific gravity of liquid}}{\text{weight in liquid} - \text{weight in air}} \quad [11]$$

The difference between the weight in air and the weight in liquid equals the weight of a volume of the liquid, which equals the volume of peas. Industry grade standards may be based on specific gravity values (Scott Rambo, personal communication, Dean Foods, Rockford, IL).

Suggested standards for frozen peas:

Fancy, 1.072 and lower
Standard, 1.073–1.084
Substandard, 1.085 and higher.

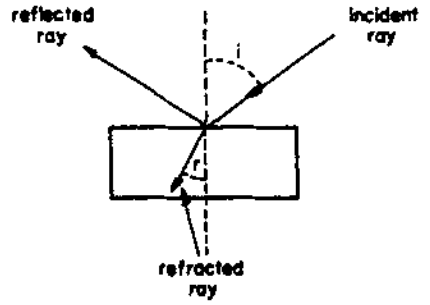
Whole kernel corn can be assayed similarly with the following specific gravity standards:

Fancy, 1.080–1.118
Reject immature, 1.079 and lower
Reject overmature, 1.119 and higher.

6.5.3 Refractometry

Moisture in liquid sugar products and condensed milks can be determined using a Baumé hydrometer (solids), a Brix hydrometer (sugar content), gravimetric means, or a **refractometer**. If it is performed correctly and no crystalline solids are evident, the refractometer procedure is rapid and surprisingly accurate (AOAC Method 9.32.14C, for solids in syrups). The refractometer has been valuable in determining the soluble solids in fruits and fruit products (AOAC Method 932.12; 976.20; 983.17).

The **refractive index** (RI) of an oil, syrup, or other liquid is a dimensionless constant that can be used to describe the nature of the food. While some refractometers are designed only to provide results as refractive indices, others, particularly hand-held, quick-to-use units, are equipped with scales calibrated to read the percentage of solids, percentage of sugars, and the like, depending on the products for which they are intended. Tables are provided with the instruments to convert values and adjust for temperature differences. Refractometers are used not just on the laboratory bench or as hand-held units. Refractometers can be installed in a liquid processing line to monitor the °Brix of products such as carbonated soft drinks, dissolved solids in orange juice, and the percentage of solids in milk (15).



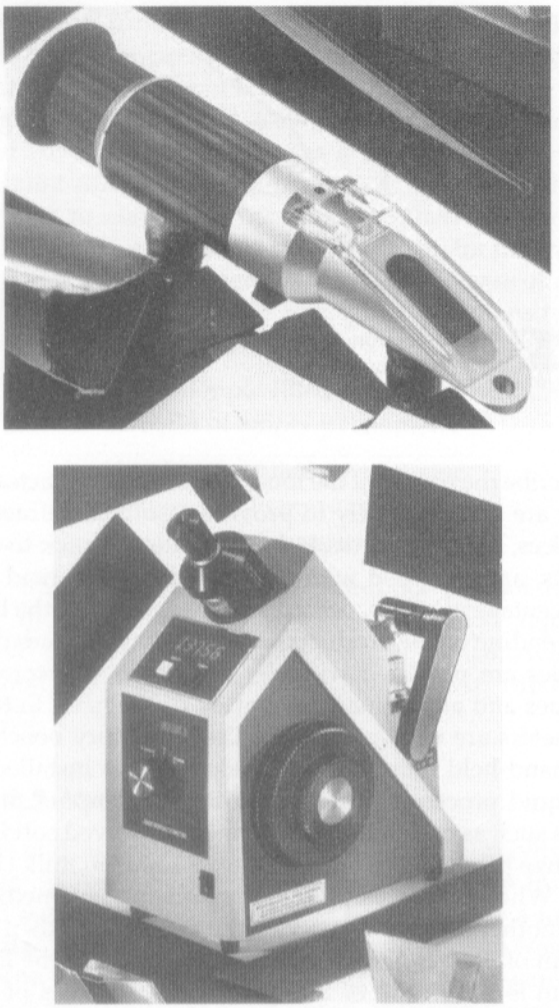
6-9 figure Reflection and refraction concepts of refractometry.

When a beam of light is passed from one medium to another and the density of the two differs, then the beam of light is bent or refracted. Bending of the light beam is a function of the media and the sines of the angles of incidence and refraction at any given temperature and pressure, and is thus a constant (Fig. 6-9). RI (η) is a ratio of the sines of the angles:

$$\eta = \frac{\text{sine incident ray angle}}{\text{sine refracted ray angle}} \quad [12]$$

All chemical compounds have an index of refraction. Therefore, this measurement can be used for the qualitative identification of an unknown compound by comparing its RI with literature values. RI varies with **concentration** of the compound, **temperature**, and **wavelength of light**. Instruments are designed to give a reading by passing a light beam of a specific wavelength through a glass prism into a liquid, the sample. Bench-top or hand-held units use **Amici prisms** to obtain the ***D* line of the sodium spectrum** or 589 nm from white light. Whenever refractive indices of standard fluids are given, these are prefaced with η_D^{20} = a value from 1.3000 to 1.7000. The Greek letter η is the symbol for RI; the 20 refers to temperature in °C; and *D* is the wavelength of the light beam, the *D* line of the sodium spectrum.

Bench-top instruments are more accurate compared to hand-held units mainly because of temperature



6-10 figure Hand-held refractometer and Abbe refractometer. (Courtesy of Cole-Parmer Instrument Company, Vernon Hills, IL.)

control (Fig. 6-10). These former units have provisions for water circulation through the head where the prism and sample meet. **Abbe refractometers** are the most popular for laboratory use. Care must be taken when cleaning the prism surface following use. Wipe the contact surface clean with lens paper and rinse with distilled water and then ethanol. Close the prism chamber and cover the instrument with a bag when not in use to protect the delicate prism surface from dust or other debris that might lead to scratches and inaccuracy.

The fact that the RI of a solution increases with concentration has been exploited in the analysis of total soluble solids of carbohydrate-based foods such as sugar syrups, fruit products, and tomato products. Because of this use, these refractometers are calibrated in °Brix (g of sucrose/100 g of sample), which is equivalent to percentage sucrose on a wt/wt basis. Refractive index measurements are used widely to approximate sugar concentration in foods, even though values are accurate only for pure sucrose solutions.

6.5.4 Infrared Analysis

Infrared spectroscopy (see Chapter 24) has attained a primary position in monitoring the composition of food products before, during, and following processing (16). It has a wide range of food applications and has proven successful in the laboratory, at-line, and on-line. Infrared spectroscopy measures the absorption of radiation (near- or mid-infrared) by molecules in foods. Different frequencies of infrared radiation are absorbed by different functional groups characteristic of the molecules in food. Similar to the use of ultraviolet (UV) or visible (Vis) light in UV–Vis spectroscopy, a sample is irradiated with a wavelength of infrared light specific for the constituent to be measured. The concentration of that constituent is determined by measuring the energy that is reflected or transmitted by the sample, which is inversely proportional to the energy absorbed. Infrared spectrometers must be calibrated for each analyte to be measured and the analyte must be uniformly distributed in the sample.

For water, near-infrared (NIR) bands (1400–1450, 1920–1950 nm) are characteristic of the—OH stretch of the water molecule and can be used to determine the moisture content of a food. NIR has been applied to moisture analysis of a wide variety of food commodities.

The use of mid-infrared milk analyzers to determine fat, protein, lactose, and total solids in milk (AOAC Method 972.16) is covered in Chapter 24 of this text. The midrange spectroscopic method does not yield moisture or solids results except by computer calculation because these instruments do not monitor at wavelengths where water absorbs. The instrument must be calibrated using a minimum of eight milk samples that were previously analyzed for fat (F), protein (P), lactose (L), and total solids (TS) by standard methods. Then, a mean difference value, a, is calculated for all samples used in calibration:

$$a = \Sigma(\text{TS} - F - P - L)/n \qquad [13]$$

where:

a= solids not measurable by the F, P, and L methods
n= number of samples
F= fat percentage
P= protein percentage
L= lactose percentage
TS= total solids percentage

Total solids then can be determined from any infrared milk analyzer results by using the formula

$$\text{TS} = a + F + P + L \qquad [14]$$

The *a* value is thus a standard value mathematically derived. Newer instruments have the algorithm in their computer software to ascertain this value automatically. Moreover, Fourier transform infrared spectroscopy (FTIR) is the latest development that allows greater flexibility in infrared assays.

6.5.5 Freezing Point

When water is added to a food product, many of the physical constants are altered. Some properties of solutions depend on the number of solute particles as ions or molecules present. These properties are vapor pressure, freezing point, boiling point, and osmotic pressure. Measurement of any of these properties can be used to determine the concentration of solutes in a solution. However, the most commonly practiced assay for milk is the change of the freezing point value. It has economic importance with regard to both raw and pasteurized milk. The **freezing point** of milk is its most constant physical property. The secretory process of the mammary gland is such that the osmotic pressure is kept in equilibrium with blood and milk. Thus, with any decrease in the synthesis of lactose, there is a compensating increase in the concentrations of Na^+ and Cl^-. While termed a physical constant, the freezing point varies within narrow limits, and the vast majority of samples from individual cows fall between −0.503°C and −0.541°C (−0.525°H and −0.565°H, temperature in °H or **Hortvet**, the surname of the inventor of the first freezing point apparatus). The average is very close to −0.517°C (−0.540°H). Herd or bulk milk will exhibit a narrower range unless the supply was watered intentionally or accidentally or if the milk is from an area where severe drought has existed. All values today are given in °C by agreement. The following is used to convert °H to °C, or °C to °H (5, 6):

$$°C = 0.9623°H - 0.0024 \quad [15]$$

$$°H = 1.03916°C + 0.0025 \quad [16]$$

The principal utility of freezing point is to measure for **added water.** However, the freezing point of milk can be altered by mastitis infection in cows and souring of milk. In special cases, nutrition and environment of the cow, stage of lactation, and processing operations for the milk can affect the freezing point. If the solute remains constant in weight and composition, the change of the freezing point varies inversely with the amount of solvent present. Therefore, we can calculate the percent H_2O added:

$$\%H_2O \text{ added} = \frac{0.517 - T}{0.517} \times 100 \quad [17]$$

where:

0.517= freezing point in °C of all milk entering a plant

T= freezing point in °C of a sample

The AOAC cryoscopic method for water added to milk (AOAC Method of 961.07) assumes a freezing point for normal milk of −0.527°C (−0.550°H). The Food and Drug Administration will reject all milk with freezing points above −0.503°C (−0.525°H). Since the difference between the freezing points of milk and water is slight and since the freezing point can be used to calculate the amount of water added, it is essential that the method be as precise as possible. The thermister used can sense temperature change to 0.001°C (0.001°H). The general technique is to supercool the solution and then induce crystallization by a vibrating reed. The temperature will rise rapidly to the freezing point or eutectic temperature as the water freezes. In the case of pure water, the temperature remains constant until all the water is frozen. In the case of milk, the temperature is read when there is no further temperature rise.

Instrumentation available is manufactured by Advanced Instruments (Fig. 6-11). Time required for the automated instruments is 1–2 min per sample using a prechilled sample.

6.6 WATER ACTIVITY

Water content alone is not a reliable indicator of food stability, since foods with the same water content differ in their perishability (17). This is at least partly due to differences in the way that water associates with other constituents in a food. Water tightly associated with other food constituents is less available for microbial growth and chemical reactions to cause decomposition. **Water activity** (a_w) is a better indication of food perishability than is water content. Water activity is defined as follows:

$$a_w = \frac{P}{P_o} \quad [18]$$

$$a_w = \frac{\text{ERH}}{100} \quad [19]$$

where:

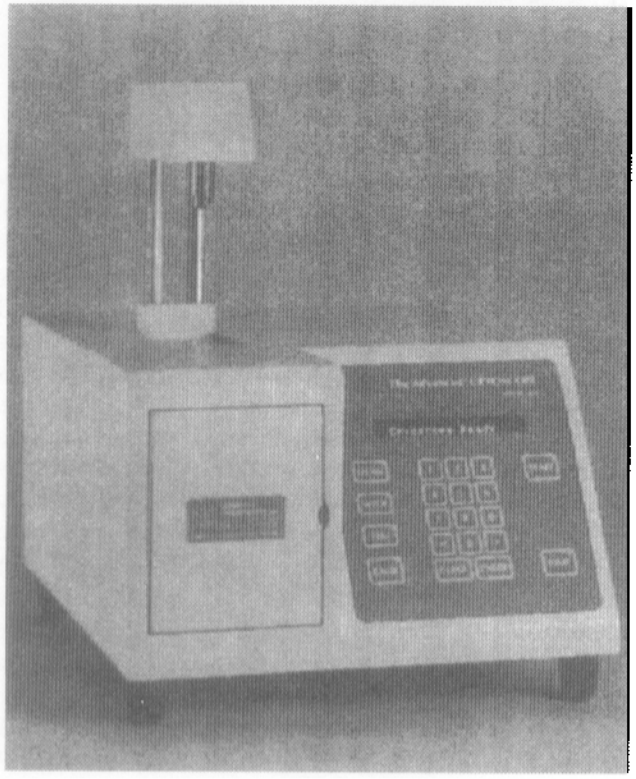
a_w = water activity

P = partial pressure of water above the sample

P_o = vapor pressure of pure water at the same temperature (specified)

ERH = equilibrium relative humidity surrounding the product

There are various techniques to measure a_w. A commonly used approach relies on measuring the amount of moisture in the equilibrated headspace above a sample of the food product, which correlates directly with



6-11 figure A model 4D3 Advanced Instruments cryoscope for freezing point determination in milk. (Courtesy of Advanced Instruments, Inc., Norwood, MA.)

sample a_w. A sample for such analysis is placed in a small closed chamber at constant temperature, and a relative humidity sensor is used to measure the ERH of the sample atmosphere after equilibration. A simple and accurate variation of this approach is the chilled mirror technique, in which the water vapor in the headspace condenses on the surface of a mirror that is cooled in a controlled manner. The dew point is determined by the temperature at which condensation takes place, and this determines the relative humidity in the headspace. Two other general approaches to measuring a_w are: (1) using the sample freezing point depression and moisture content to calculate a_w and (2) equilibrating a sample in a chamber held at constant relative humidity (by means of a saturated salt solution) and then using the water content of the sample to calculate a_w (17).

6.7 COMPARISON OF METHODS

6.7.1 Principles

Oven drying methods involve the removal of moisture from the sample and then a weight determination of the solids remaining to calculate the moisture content. Nonwater volatiles can be lost during drying, but their loss is generally a negligible percentage of the amount of water lost. Distillation procedures also involve a separation of the moisture from the solids, and the moisture is quantitated directly by volume. Karl Fischer titration is based on chemical reactions of the moisture present, reflected as the amount of titrant used.

Dielectric and conductivity methods are based on electrical properties of water. Hydrometric methods are based on the relationship between specific gravity and moisture content. The RI method is based on how water in a sample affects the refraction of light. Near-infrared analysis of water in foods is based on measuring the absorption at wavelengths characteristic of the molecular vibration in water. Freezing point is a physical property of milk that is changed by a change in solute concentration.

6.7.2 Nature of Sample

While most foods will tolerate oven drying at high temperatures, some foods contain volatiles that are lost at such temperatures. Some foods have constituents that undergo chemical reactions at high temperatures to generate or utilize moisture or other compounds, to affect the calculated moisture content. Vacuum oven drying at reduced temperatures may overcome such problems for some foods. However, a distillation technique is necessary for some food to minimize volatilization and decomposition. For foods very low in moisture or high in fats and sugars, Karl Fischer titration is often the method of choice. The use of a pycnometer, hydrometer, and refractometer requires liquid samples, ideally with limited constituents.

6.7.3 Intended Purposes

Moisture analysis data may be needed quickly for quality control purposes, in which high accuracy may not be necessary. Of the oven drying methods, microwave drying, infrared drying, and the moisture analyzer technique are fastest. Some forced draft oven procedures require less than 1 hr drying, but most forced draft oven and vacuum oven procedures require a much longer time. The electrical, hydrometric, and RI methods are very rapid but often require correlation to less empirical methods. Oven drying procedures are official methods for a variety of food products. Reflux distillation is an AOAC method for chocolate, dried vegetables, dried milk, and oils and fats. Such official methods are used for regulatory and nutrition labeling purposes.

6.8 SUMMARY

The moisture content of foods is important to food processors and consumers for a variety of reasons. While moisture determination may seem simplistic, it is often one of the most difficult assays in obtaining accurate and precise results. The free water present in food is generally more easily quantitated as compared to the adsorbed moisture and the water of hydration. Some moisture analysis methods involve a separation of moisture in the sample from the solids and then quantitation by weight or volume. Other methods do not involve such a separation but instead are based on some physical or chemical property of the water in the sample. A major difficulty with many methods is attempting to remove or otherwise quantitate all water present. This often is complicated by decomposition or interference by other food constituents. For each moisture analysis method, there are factors that must be controlled or precautions that must be taken to ensure accurate and precise results. Careful sample collection and handling procedures are extremely important and cannot be overemphasized. The choice of moisture analysis method is often determined by the expected moisture content, nature of other food constituents (e.g., highly volatile, heat sensitive), equipment available, speed necessary, accuracy and precision required, and intended purpose (e.g., regulatory or in-plant quality control).

6.9 STUDY QUESTIONS

1. Identify five factors that one would need to consider when choosing a moisture analysis method for a specific food product.
2. Why is standardized methodology needed for moisture determinations?
3. What are the potential advantages of using a vacuum oven rather than a forced draft oven for moisture content determination?
4. In each case specified below, would you likely overestimate or underestimate the moisture content of a food product being tested? Explain your answer.
   a. forced draft oven:
      - particle size too large
      - high concentration of volatile flavor compounds present
      - lipid oxidation
      - sample very hygroscopic
      - alteration of carbohydrates (e.g., Maillard browning)
      - sucrose hydrolysis
      - surface crust formation
      - splattering
      - desiccator with dried sample not sealed properly

   b. toluene distillation:
      - emulsion between water in sample and solvent not broken
      - water clinging to condenser

   c. Karl Fischer:
      - very humid day when weighing original samples
      - glassware not dry
      - sample ground coarsely
      - food high in vitamin C
      - food high in unsaturated fatty acids
5. The procedure for an analysis for moisture in a liquid food product requires the addition of 1–2 ml of deionized water to the weighed sample in the moisture pan. Why should you add water to an analysis in which moisture is being determined?
6. A new instrument based on infrared principles has been received in your laboratory to be used in moisture analysis. Briefly describe the way you would ascertain if the new instrument would meet your satisfaction and company standards.
7. A technician you supervise is to determine the moisture content of a food product by the Karl Fischer method. Your technician wants to know what is this "Karl Fischer reagent water equivalence" that is used in the equation to calculate percentage of moisture in the sample, why is it necessary, and how is it determined. Give the technician your answer.
8. To explain and contrast the principles (not procedures) in determining the moisture content of food products by the following method, complete the table below. (Assume that sample selection and handling has been done appropriately.)

	What is Actually Measured?	*How is Water Removed/ Reacted/ Identified?*	*What Assumptions are Made in Trusting the Data Obtained (or Precautions Taken to Ensure Accurate Data)?*
Microwave oven			
NIR			
Karl Fischer			
Toluene distillation			

9. You are fortunate to have available in your laboratory the equipment for doing moisture analysis by essentially all methods—both official and rapid quality control methods. For each of the food products listed below (with the purpose specified as rapid quality control or official), indicate (a) the name of the method you would use, (b) the principle (not procedure) for the method, (c) a justification for use of that method (as compared to using a hot air drying oven), and (d) two cautions in use of the method to ensure accurate results.
   a. ice cream mix (liquid)—quality control
   b. milk chocolate—official
   c. spices—official

d. syrup for canned peaches—quality control
e. oat flour—quality control

10. You are a manufacturer of processed cheese. The maximum allowed moisture content for your product is 40%. Your current product has a mean moisture content of 38%, with a standard deviation of 0.7. It would be possible to increase your mean moisture content to 39.5% if you could reduce your standard deviation to 0.25. This would result in a saving of $3.4 million per year. You can accomplish this by rapidly analyzing the moisture content of the cheese blend prior to the cooking step of manufacture. The cheese blend is prepared in a batch process, and you have 10 min to adjust the moisture content of each batch.
    a. Describe the rapid moisture analysis method you would use. Include your rationale for selecting the method.
    b. How would you ensure the accuracy and precision of this method (you need to be sure your standard deviation is below 0.25)?
11. You work in a milk drying plant. As part of the production process, you need to rapidly analyze the moisture content of condensed milk.
    a. What rapid secondary method would you use, and what primary method would you use to calibrate the secondary method? Additionally, how would you ensure the accuracy and precision of your secondary method?
    b. Your results with the secondary method are consistently high (1%), based on the secondary method you chose. What are some potential problems, and how would you correct them?
12. During a 12-hr period, 1000 blocks (40 lbs each) from 10 different vats (100 blocks per vat) of Cheddar cheese were produced. It was later realized that the cooking temperature was too low during cheesemaking. You are concerned that this might increase the moisture content of the cheese above the legal requirement. Describe the sampling plan and method of analysis you would use to determine the moisture content of the cheese. You want the results within 48 hr so you can determine what to do with the cheese.

6.10 PRACTICE PROBLEMS

1. As an analyst, you are given a sample of condensed soup to analyze to determine if it is reduced to the correct concentration. By gravimetric means, you find that the concentration is 26.54% solids. The company standard reads 28.63%. If the starting volume were 1000 gallons at 8.67% solids, and the weight is 8.5 pounds per gallon, how much more water must be removed?
2. Your laboratory just received several sample containers of peas to analyze for moisture content. There is a visible condensate on the inside of the container. What is your procedure to obtain a result?
3. You have the following gravimetric results: weight of dried pan and glass disk = 1.0376 g, weight of pan and liquid sample 4.6274 g, and weight of the pan and dried sample 1.7321 g. What was the moisture content of the sample and what is the percent solids?

Answers

1. The weight of the soup initially is superfluous information. By condensing the soup to 26.54% solids from 8.67% solids, the volume is reduced to 326.7 gallons [(8.67/26.54) × 1000]. You need to reduce the volume further to obtain 28.63% solids [(8.67/28.63) × 1000], or 302.8 gallons. The difference in the gallons obtained is 23.9, or the volume of water that must be removed from the partially condensed soup to comply with company standards (326.7 − 302.8).
2. This problem focuses on a real issue in the food processing industry—when do you analyze a sample and when don't you? It would appear that the peas have lost moisture that should be within the vegetable for correct results. You will need to grind the peas in a food mill or blender. If the peas are in a Mason jar or one that fits a blender head, no transfer is needed. Blend the peas to a creamy texture. If a container transfer was made, then put the blended peas back into the original container. Mix with the residual moisture to a uniform blend. Collect a sample for moisture analysis. You should note on the report form containing the results of the analysis that the pea samples had free moisture on container walls when they arrived.
3. Note Equations [2]–[4] in section 6.2.1.7. To use any of the equations, you must subtract the weight of the dried pan and glass disc. Then you obtain 3.5898 g of original sample and 0.6945 g when dried. By subtracting these results, you have water removed or 2.8953 g. Then (0.6945/3.5898) × 100 = 19.35% solids and (2.8953/3.5898) × 100 = 80.65% water.

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7

chapter

Ash Analysis

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7.1 INTRODUCTION

Ash refers to the inorganic residue remaining after either ignition or complete oxidation of organic matter in a foodstuff. A basic knowledge of the characteristics of various ashing procedures and types of equipment is essential to ensure reliable results. Two major types of ashing are used: dry ashing, primarily for proximate composition and for some types of specific mineral analyses; wet ashing (oxidation), as a preparation for the analysis of certain minerals. Microwave systems now are available for both dry and wet ashing, to speed the processes. Most dry samples (i.e., whole grain, cereals, dried vegetables) need no preparation, while fresh vegetables need to be dried prior to ashing. High-fat products such as meats may need to be dried and fat extracted before ashing. The ash content of foods can be expressed on either a wet weight (as is) or on a dry weight basis.

7.1.1 Definitions

Dry ashing refers to the use of a muffle furnace capable of maintaining temperatures of 500–600°C. Water and volatiles are vaporized and organic substances are burned in the presence of oxygen in air to CO_2, and oxides of N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides, and silicates. Elements such as Fe, Se, Pb, and Hg may partially volatilize with this procedure, so other methods must be used if ashing is a preliminary step for specific elemental analysis.

Wet ashing is a procedure for oxidizing organic substances by using acids and oxidizing agents or their combinations. Minerals are solubilized without volatilization. Wet ashing often is preferable to dry ashing as a preparation for specific elemental analysis. Wet ashing often uses a combination of acids, and requires a special perchloric acid hood if that acid is used.

7.1.2 Importance of Ash in Food Analysis

Ash content represents the total mineral content in foods. Determining the ash content may be important for several reasons. It is a part of the proximate analysis for nutritional evaluation. Ashing is the first step in the preparation of a food sample for specific elemental analysis. Because certain foods are high in particular minerals, ash content becomes important. We can usually expect a constant elemental content from the ash of animal products, but that from plant sources is variable.

7.1.3 Ash Contents in Foods

The average ash content of the various food groups is given in Table 7-1. The ash content of most fresh foods rarely is greater than 5%. Pure oils and fats generally contain little or no ash, products such as cured bacon may contain 6% ash, and dried beef may be as high as 11.6% (wet weight basis).

Fats, oils, and shortenings vary from 0.0 to 4.09% ash, while dairy products vary from 0.5 to 5.1%. Fruits, fruit juice, and melons contain 0.2–0.6% ash, while dried fruits are higher (2.4–3.5%). Flours and meals vary from 0.3 to 1.4% ash. Pure starch contains 0.3% and wheat germ 4.3% ash. It would be expected that grain and grain products with bran would tend to be higher in ash content than such products without bran. Nuts and nut products contain 0.8–3.4% ash, while meat, poultry, and seafoods contain 0.7–1.3% ash.

7-1 table **Ash Content of Selected Foods**

Food Item	*Percent Ash (Wet Weight Basis)*
Cereals, bread, and pasta	
Rice, brown, long-grain, raw	1.5
Corn meal, whole-grain, yellow	1.1
Hominy, canned, white	0.9
White rice, long-grain, regular, raw, enriched	0.6
Wheat flour, whole-grain	1.6
Macaroni, dry, enriched	0.7
Rye bread	2.5
Dairy products	
Milk, whole, fluid	0.7
Evaporated milk, whole	1.6
Butter, with salt	2.1
Cream, fluid, half and half	0.7
Margarine, hard, regular, soybean	2.0
Yogurt, plain, low fat	1.0
Fruits and vegetables	
Apples, raw, with skin	0.3
Bananas, raw	0.8
Cherries, sweet, raw	0.5
Raisins	1.8
Potatoes, raw, skin	1.6
Tomatoes, red, ripe, raw	0.4
Meat, poultry, and fish	
Eggs, whole, raw, fresh	0.9
Fish fillet, battered or breaded, and fried	2.5
Pork, fresh, leg (ham), whole, raw	0.9
Hamburger, regular, single patty, plain	1.7
Chicken, broilers or fryers, breast meat only, raw	1.0
Beef, chuck, arm pot roast, raw	0.9

From USDA Nutrient Database for Standard Reference. Release 14 (July 2001) http://www.nal.usda.gov/fnic/cgi bin/nut_search.pl

7.2 METHODS

Principles, materials, instrumentation, general procedures, and applications are described below for various

ash determination methods. Refer to methods cited for detailed instructions of the procedures.

7.2.1 Sample Preparation

It cannot be overemphasized that the small sample used for ash, or other determinations, needs to be very carefully chosen so that it represents the original materials. A 2–10-g sample generally is used for ash determination. For that purpose, milling, grinding, and the like probably will not alter the ash content much; however, if this ash is a preparatory step for specific mineral analyses, contamination by microelements is of potential concern. Remember, most grinders and mincers are of steel construction. Repeated use of glassware can be a source of contaminants as well. The water source used in dilutions also may contain contaminants of some microelements. Distilled-deionized water should be used.

7.2.1.1 Plant Materials

Plant materials are generally dried by routine methods prior to grinding. The temperature of drying is of little consequence for ashing. However, the sample may be used for multiple determinations—protein, fiber, and so on—which require consideration of temperature for drying. Fresh stem and leaf tissue probably should be dried in two stages (i.e., first at a lower temperature of 55°C, then a higher temperature) especially to prevent artifact lignin. Plant material with 15% or less moisture may be ashed without prior drying.

7.2.1.2 Fat and Sugar Products

Animal products, syrups, and spices require treatments prior to ashing because of high fat and moisture (spattering, swelling) or high sugar content (foaming) that may result in loss of sample. Meats, sugars, and syrups need to be evaporated to dryness on a steam bath or with an infrared (IR) lamp. One or twodrops of olive oil (which contains no ash) are added to allow steam to escape as a crust is formed on the product.

Smoking and burning may occur upon ashing for some products (e.g., cheese, seafood, spices). Allow this smoking and burning to finish slowly by keeping the muffle door open prior to the normal procedure. A sample may be ashed after drying and fat extraction. In most cases, mineral loss is minimal during drying and fat extraction. Under no circumstances should fat-extracted samples be heated until all the ether has been evaporated.

7.2.2 Dry Ashing

7.2.2.1 Principles and Instrumentation

Dry ashing is incineration at high temperature (525°C or higher). Incineration is accomplished with a muffle furnace. Several models of muffle furnaces are available, ranging from large-capacity units requiring either 208 or 240 voltage supplies to small bench-top units utilizing 110-V outlets.

Crucible selection becomes critical in ashing because type depends upon the specific use. **Quartz crucibles** are resistant to acids and halogens, but not alkali, at high temperatures. **Vycor® brand crucibles** are stable to 900°C, but **Pyrex® Gooch crucibles** are limited to 500°C. Ashing at a lower temperature of 500–525°C may result in slightly higher ash values because of less decomposition of carbonates and loss of volatile salts. **Porcelain crucibles** resemble quartz crucibles in their properties but will crack with rapid temperature changes. Porcelain crucibles are relatively inexpensive and usually the crucible of choice. **Steel crucibles** are resistant to both acids and alkalies and are inexpensive, but they are composed of chromium and nickel, which are possible sources of contamination. **Platinum crucibles** are very inert and are probably the best crucibles but they are currently far too expensive for routine use for large numbers of samples. **Quartz fiber crucibles** are disposable, unbreakable, and can withstand temperatures up to 1000°C. They are porous, allowing air to circulate around the sample and speed combustion. This reduces ashing times significantly and makes them ideal for solids and viscous liquids. Quartz fiber also cools in seconds, virtually eliminating the risk of burns.

All crucibles should be **marked for identification**. Marks on crucibles with a felt-tip marking pen will disappear during ashing in a muffle furnace. Laboratory inks scribed with a steel pin are available commercially. Crucibles also may be etched with a diamond point and marked with a 0.5 *M* solution of $FeCl_3$ in 20% HCl. An iron nail dissolved in concentrated HCl forms a brown goo that is a satisfactory marker. The crucibles should be fired and cleaned prior to use.

The *advantages* of conventional dry ashing are that it is a safe method, it requires no added reagents or blank subtraction, and little attention is needed once ignition begins. Usually a large number of crucibles can be handled at once, and the resultant ash can be used for such other analyses as most individual elements, acid-insoluble ash, and water-soluble and insoluble ash. The *disadvantages* are the length of time required (12–18 hr, or overnight) and expensive equipment. There will be a loss of the volatile elements and interactions between mineral components and crucibles. Volatile elements at risk of being lost include As, B, Cd, Cr, Cu, Fe, Pb, Hg, Ni, P, V, and Zn.

7.2.2.2 Procedures

AOAC International has several dry ashing procedures (e.g., AOAC Methods 900.02 A or B, 920.117, 923.03) for certain individual foodstuffs.

The general procedure includes the following steps:

1. Weigh a 5–10-g sample into a tared crucible. Predry if the sample is very moist.
2. Place crucibles in cool muffle furnace. Use tongs, gloves, and protective eyeware if the muffle furnace is warm.
3. Ignite 12–18 hr (or overnight) at about 550°C.
4. Turn off muffle furnace and wait to open it until the temperature has dropped to at least 250°C, preferably lower. Open door carefully to avoid losing ash that may be fluffy.
5. Using safety tongs, quickly transfer crucibles to a desiccator with a porcelain plate and desiccant. Cover crucibles, close desiccator, and allow crucibles to cool prior to weighing.

Note: Warm crucibles will heat air within the desiccator. With hot samples, a cover may bump to allow air to escape. A vacuum may form on cooling. At the end of the cooling period, the desiccator cover should be removed gradually by sliding to one side to prevent a sudden inrush of air. Covers with a ground glass sleeve or fitted for a rubber stopper allow for slow release of a vacuum.

The ash content is calculated as follows:

$$\%\ \text{ash (dry basis)} = \frac{\text{wt after ashing} - \text{tare wt of crucible}}{\text{original sample wt} \times \text{dry matter coefficient}} \times 100 \quad [1]$$

where:

$$\text{dry matter coefficient} = \%\ \text{solids}/100$$

For example, if corn meal is 87% dry matter, the dry matter coefficient would be 0.87. If ash is calculated on an as-received or wet weight basis (includes moisture), delete the dry matter coefficient from the denominator. If moisture was determined in the same crucible prior to ashing, the denominator becomes (dry sample wt − tared crucible wt).

7.2.2.3 Special Applications

Some of the AOAC procedures recommend steps in addition to those listed previously. If carbon is still present following the initial incineration, several drops of water or nitric acid should be added; then the sample should be re-ashed. If the carbon persists, such as with high-sugar samples, follow this procedure:

1. Suspend the ash in water.
2. Filter through ashless filter paper because this residue tends to form a glaze.
3. Dry the filtrate.
4. Place paper and dried filtrate in muffle furnace and re-ash.

Other suggestions that may be helpful and accelerate incineration:

1. High-fat samples should be extracted either by using the crude fat determination procedure or by burning off prior to closing the muffle furnace. Pork fat, for example, can form a combustible mixture inside the furnace and burn with the admission of oxygen if the door is opened.
2. Glycerin, alcohol, and hydrogen will accelerate ashing.
3. Samples such as jellies will spatter and can be mixed with cotton wool.
4. Salt-rich foods may require a separate ashing of water-insoluble components and salt-rich water extract. Use a crucible cover to prevent spattering.
5. An alcoholic solution of magnesium acetate can be added to accelerate ashing of cereals. An appropriate blank determination is necessary.

7.2.3 Wet Ashing

7.2.3.1 Principle, Materials, and Applications

Wet ashing is sometimes called **wet oxidation** or **wet digestion**. Its primary use is preparation for specific mineral analysis and metallic poisons. Often, analytical testing laboratories use only wet ashing in preparing samples for certain mineral analyses (e.g., Fe, Cu, Zn, P), because losses would occur by volatilization during dry ashing.

There are several *advantages* to using the wet ashing procedure. Minerals will usually stay in solution, and there is little or no loss from volatilization because of the lower temperature. The oxidation time is short and requires a hood, hot plate, and long tongs, plus safety equipment.

The *disadvantages* of wet ashing are that it takes virtually constant operator attention, corrosive reagents are necessary, and only small numbers of samples can be handled at any one time. If the wet digestion utilizes perchloric acid, all work needs to be carried out in an expensive special fume hood called a **perchloric acid hood**.

Unfortunately, a single acid used in wet ashing does not give complete and rapid oxidation of organic

material, so a mixture of acids often is used. Combinations of the following acid solutions are used most often: (1) **nitric acid**, (2) **sulfuric acid/hydrogen peroxide**, and (3) **perchloric acid**. Different combinations are recommended for different types of samples. The nitric-perchloric combination is generally faster than the sulfuric-nitric procedure. While wet digestion with perchloric acid is an AOAC procedure (e.g., AOAC Method 975.03), many analytical laboratories avoid if possible the use of perchloric acid in wet ashing, and instead use a combination of nitric acid with either sulfuric acid, hydrogen peroxide, or hydrochloric acid.

Wet oxidation with perchloric acid is ***extremely*** dangerous since the perchloric acid has a tendency to explode. The perchloric acid hood that must be used has wash-down capabilities and does not contain plastic or glycerol-base caulking compounds. Precautions for use of perchloric acid are found in the AOAC methods under "Safe Handling of Special Chemical Hazards." Cautions must be taken when fatty foods are wet ashed using perchloric acid. While perchloric acid does not interfere with atomic absorption spectroscopy, it does interfere in the traditional colorimetric assay for iron by reacting with iron in the sample to form ferrous perchlorate, which forms an insoluble complex with the o-phenanthrolene in the procedure.

7.2.3.2 Procedures

The following is a wet ash procedure using concentrated nitric and sulfuric acids (***to be performed in a fume hood***) (W. G. Ikins, Silliker Laboratories, Chicago, IL, personal communication):

1. Accurately weigh a dried, ground 1-g sample in a 125-ml Erlenmeyer flask (previously acid washed and dried).
2. Prepare a blank of 3 ml H_2SO_4 and 5 ml HNO_3, to be treated like the samples. (Blank is to be run with every set of samples.)
3. Add 3 ml H_2SO_4 followed by 5 ml HNO_3 to the sample in the flask.
4. Heat the sample on a hot plate at ca. 200°C (boiling). Brown-yellow fumes will be observed.
5. Once the brown-yellow fumes cease and white fumes from decomposing H_2SO_4 are observed, the sample will become darker. Remove the flask from the hot plate. Do not allow the flask to cool to room temperature.
6. *Slowly* add 3–5 ml HNO_3.
7. Put the flask back on the hot plate and allow the HNO_3 to boil off. Proceed to the next step when all the HNO_3 is removed and the color is clear to straw yellow. If the solution is still dark in color, add another 3–5 ml HNO_3 and boil. Repeat the process until the solution is clear to straw yellow.
8. Allow the sample to cool to room temperature, then quantitatively transfer the sample to a 10-ml volumetric flask.
9. Dilute the sample to volume with ultrapure water, and mix well. Dilute further, as appropriate, for the specific type of mineral being analyzed.

The following procedure for a modified dry-wet ash oxidation may be used. It is listed under "Minerals in Ready-to-Feed Milk-Based Infant Formula" (AOAC Method 985.35).

1. Evaporate moist samples (25–50 ml) at 100°C overnight or in a microwave drying oven.
2. Heat on a hot plate until smoking ceases.
3. Ash at 525°C for 3–5 hr.
4. Cool and wet with deionized distilled water plus 0.5–3.0 ml HNO_3.
5. Dry on a hot plate or steam bath and incinerate at 525°C for 1–2 hr.
6. Weigh sample after cooling in a desiccator.
7. Repeat steps 4 and 5 if carbon persists. (Caution: Some K may be lost with repeated ashing.)

7.2.4 Microwave Ashing

Both **wet ashing** and **dry ashing** can be done using microwave instrumentation, rather than the conventional dry ashing in a muffle furnace and wet ashing in a flask or beaker on a hot plate. The CEM Corporation (Matthews, NC) has developed a series of instruments for dry and wet ashing, as well as other laboratory systems for microwave-assisted chemistry. While the ashing procedures by conventional means can take many hours, the use of microwave instrumentation can reduce sample preparation time to minutes, allowing laboratories to increase their sample throughput significantly. This advantage has led to widespread use of microwave ashing, especially for wet ashing, both within analytical laboratories and quality control laboratories within food companies.

7.2.4.1 Microwave Wet Ashing

Microwave wet ashing (acid digestion) may be performed safely in either an open- or closed-vessel microwave system. Choice of the system depends on the amount of sample and the temperatures required to digest it. Due to the ability of the closed vessels to contain higher pressures (some vessels can handle up to 1500 psi), acids may be heated past their boiling points. This ensures a more complete dissolution of hard-to-digest substances. It also allows the chemist to use nitric acid with samples that might normally

require a harsher acid, such as sulfuric or perchloric. In closed vessels specifically designed for high-temperatures/high-pressure reactions, nitric acid can reach a temperature of 240°C. Thus, **nitric acid** is often the acid of choice, though hydrochloric, hydrofluoric, and sulfuric acids also are used, depending on the sample and the subsequent analysis being performed. **Closed-vessel microwave digestion systems** can process up to 14 samples at a time, with vessel liners available in Teflon®, TFM™ Fluoropolymer, and quartz. These systems allow the input of time, temperature and pressure parameters in a step-by-step format (ramping). In addition, some instruments enable the user to adjust the power and offer "change-on-the-fly" software, which allows the method to be changed while the reaction is running.

Typically, in a closed-vessel microwave system, sample is placed in vessels with the appropriate amount of acid. The vessels are sealed, and set on a carousel where the temperature and pressure sensors are connected to a control vessel. The carousel then is placed in the microwave cavity and the sensors connected to the instrument. Time, temperature, pressure, and power parameters are chosen and the unit is started. Digestions normally take less than 30 min. Because of the pressure generated by raising the temperature of a reaction, vessels must be allowed to cool before being opened. The ability to process multiple samples simultaneously provides the chemist with greater throughput than traditional methods. (Note that some closed-vessel microwave digestion systems may also be used for acid concentration, solvent extraction, protein hydrolysis, and synthesis with the proper accessories.)

Open-vessel digestion systems are used often for larger sample sizes (up to 10 g) and for samples that generate substantial amounts of gas as they are digested. Open-vessel systems can process up to six samples, each according to its own parameters in a sequential or simultaneous format. Teflon®, quartz, or Pyrex® vessels are used and condensers are added for refluxing. Acid (reagent) is automatically added according to the programmed parameters. Sulfuric and nitric acids are used most often with open-vessel systems, as they process reactions under atmospheric conditions; however, hydrochloric and hydrofluoric acids, as well as hydrogen peroxide, can be used. These instruments do not require the use of a fume hood, because a vapor containment system contains and neutralizes harmful fumes.

Generally, in an open-vessel microwave system, sample is placed in a vessel and the vessel set in a slot in the microwave system. Time, temperature, and reagent addition parameters are then chosen. The unit is started, the acid is added, and the vapor containment system neutralizes the fumes from the reaction. Samples are typically processed much faster and more reproducibly than on a conventional hot plate. (Note that some open-vessel systems may be used for evaporation and acid concentration as well.)

7.2.4.2 Microwave Dry Ashing

Compared to conventional dry ashing in a muffle furnace that often takes many hours, **microwave muffle furnaces** can ash samples in minutes, decreasing analysis time by as much as 97%. Microwave muffle furnaces can reach temperatures of up to 1200°C. These systems may be programmed with various methods and to automatically warm up and cool down. In addition, they are equipped with exhaust systems that circulate the air in the cavity to help decrease ashing times. Some also have scrubber systems to neutralize any fumes. Any crucible that may be used in a conventional muffle furnace may be used in a microwave furnace, including those made of porcelain, platinum, quartz, and quartz fiber. Quartz fiber crucibles cool in seconds and are not breakable. Some systems can process up to 15 (25-ml) crucibles at a time.

Typically, in microwave dry ashing, a desiccated crucible is weighed and then sample is added and it is weighed again. The crucible then is placed in the microwave furnace and the time and temperature parameters are set. A step-by-step (ramping) format may be used when programming the method. The system is started and the program is run to completion. The crucible then is carefully removed with tongs and reweighed. The sample then may be further analyzed, if necessary. Some tests call for acid to be added to a dry ashed sample, which is then digested for further analysis.

A comparative study (9) showed that dry ashing various plants for 40 min using a microwave system (CEM Corporation, Matthews NC) was similar to the 4-hr time in a conventional muffle furnace. Twenty minutes was shown to be adequate for the plant material used except for Cu determinations, which needed 40 min to obtain similar results. Other comparative examples include dried egg yolks which can be ashed in 20 min in a microwave system, but require 4 hr in a conventional muffle furnace. It takes 16 hr to ash lactose in a conventional muffle furnace, but only 35 min in a microwave furnace. Though microwave furnaces may not hold as many samples as a conventional furnace, their speed actually allows significantly more samples to be processed in the same amount of time. Also, microwave furnaces do not require fume hood space.

7.2.5 Other Ash Measurements

The following are several special ash measurements and their applications:

1. **Soluble and Insoluble Ash** (e.g., AOAC Method 900.02)—Applied to fruits.

2. **Ash Insoluble in Acid**—A measure of the surface contamination of fruits and vegetables and wheat and rice coatings; contaminants are generally silicates and remain insoluble in acid, except HBr.
3. **Alkalinity of Ash** (e.g., AOAC Method 900.02, 940.26)—Ash of fruits and vegetables is alkaline; ash of meats and some cereals is acid.
4. **Sulfated Ash** (AOAC Method 900.02, 950.77)—Applied to sugars, syrups, and color additives.

7.3 COMPARISON OF METHODS

Ash determination by dry ashing requires expensive equipment, especially if a large number of samples is analyzed. The muffle furnace may have to be placed in a heat room along with drying ovens and it requires a 220-V outlet. It is important to make sure large furnaces of that type are equipped with a double-pole, single-throw switch. Heating coils are generally exposed, and care must be taken when taking samples in and out with metal tongs. Desk-top furnaces (110 V) are available for fewer samples. Wet ashing requires a hood (a special hood if perchloric acid is used), corrosive reagents, and constant operator attention. While wet oxidation causes little volatilization, dry ashing will result in the loss of volatile elements. The type of further elemental analyses will dictate the equipment. Some micro- and most volatile elements will require special equipment and procedures. Refer to Chapters 12 and 25 for specific preparation procedures for elemental analyses. Both dry and wet ashing can be done using microwave systems, that utilize relatively expensive instrumentation, but they greatly reduce the time for ashing and do not require use of a fume hood.

7.4 SUMMARY

The two major types of ashing, dry ashing and wet oxidation (ashing), both can be done by conventional means or using microwave systems. The procedure of choice depends upon the use of ash following its determination, and limitations based on cost, time, and sample numbers. Conventional dry ashing is based upon incineration at high temperatures in a muffle furnace. Except for certain elements, the residue may be used for further specific mineral analyses. Wet ashing (oxidation) often is used as a preparation for specific elemental analysis by simultaneously dissolving minerals and oxidizing all organic material. Wet ashing conserves volatile element but requires more operator time than dry ashing, and is limited to a smaller number of samples. Dry and wet ashing using microwave technology reduces the time for analyses and requires little additional equipment (special fume hood) or space (heat room).

7.5 STUDY QUESTIONS

1. Identify four potential sources of error in the preparation of samples for ash analysis, and describe a way to overcome each.
2. You are determining the total ash content of a product using the conventional dry ashing method. Your boss asks you to switch to a conventional wet ashing method because he/she has heard it takes less time than dry ashing.
   a. Do you agree or disagree with your boss concerning the time issue, and why?
   b. Not considering the time issues, why might you want to continue using dry ashing, *and* why might you change to wet ashing?
3. Your lab technician was to determine the ash content of buttermilk by conventional dry ashing. The technician weighed 5 g of buttermilk into one weighed platinum crucible, immediately put the crucible into the muffle furnace using a pair of all stainless steel tongs, and ashed the sample for 48 hr at 800°C. The crucible was removed from the muffle furnace and set on a rack in the open until it was cool enough to reweigh. Itemize the instructions you should have given your technician before beginning, so there would not have been the mistakes made as described above.
4. How would you recommend to your technician to overcome the following problems that could arise in conventional dry ashing of various foods?
   a. You seem to be getting volatilization of phosphorus, when you want to later determine the phosphorus content.
   b. You are getting incomplete combustion of a product high in sugar after a typical dry ashing procedure (i.e., the ash is dark colored, not white or pale gray).
   c. The typical procedure takes too long for your purpose. You need to speed up the procedure, but you do not want to use the standard wet ashing procedure.
   d. You have reason to believe the compound you want to measure after dry ashing may be reacting with the porcelain crucibles being used.
   e. You want to determine the iron content of some foods but cannot seem to get the iron solubilized after the dry ashing procedure.
5. Identify an advantage and disadvantage of using microwave wet digesters or microwave muffle furnaces compared to conventional units.

7.6 PRACTICE PROBLEMS

1. A grain was found to contain 11.5% moisture. A 5.2146-g sample was placed into a crucible (28.5053 g tare). The ashed crucible weighed 28.5939 g. Calculate the percentage ash on (a) an as-received basis and (b) a dry matter basis.
2. A vegetable (23.5000 g) was found to have 0.0940 g acid-insoluble ash. What is the percentage acid-insoluble ash?

3. You wish to have at least 100 mg ash from a cereal grain. Assuming 2.5% ash on average, how many grams of the grain should be weighed for ashing?
4. You wish to have a coefficient of variation (CV) below 5% with your ash analyses. The following ash data are obtained: 2.15%, 2.12%, 2.07%. Are these data acceptable, and what is the CV?
5. The following data were obtained on a sample of hamburger: sample wt, 2.034 g; wt after drying, 1.0781 g; wt after ether extraction, 0.4679 g; and wt of ash, 0.0233 g. What is the percentage ash on (a) a wet weight basis and (b) a fat-free basis?

Answers

1. (a) 1.70%, (b) 1.92%
2. 0.4%
3. 4 g
4. Yes, 1.9%
5. (a) 1.1%, (b) 1.57%

7.7 RESOURCE MATERIALS

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5. Pomeranz, Y., and Meloan, C. 1994. *Food Analysis: Theory and Practice*, 3rd ed. Chapman & Hall, New York. Chapter 35 on ash and minerals gives an excellent narrative on ashing methods and is easy reading for a student in food chemistry. A good reference list of specific mineral losses is given at the end of the chapter. No stepwise procedures are given.
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8. Wooster, H.A. 1956. *Nutritional Data*, 3rd ed. H.J. Heinz Co., Pittsburgh, PA.
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8.1 INTRODUCTION

8.1.1 Definitions

Lipids, proteins, and carbohydrates constitute the principal structural components of foods. Lipids are a group of substances that, in general, are soluble in ether, chloroform, or other organic solvents but are sparingly soluble in water. However, there exists no clear definition of a lipid, primarily due to the water solubility of certain molecules that fall within one of the variable categories of food lipids (1). Some lipids, such as triacylglycerols, are very hydrophobic. Other lipids, such as di- and monoacylglycerols, have both hydrophobic and hydrophilic moieties in their molecules and are soluble in relatively polar solvents (2). Short-chain fatty acids such as C1–C4 are completely miscible in water and insoluble in nonpolar solvents (1). However, the most widely accepted definition is based on solubility as previously stated. While most macromolecules are characterized by common structural features, the designation of "lipid" being defined by solubility characteristics is unique to lipids (2). Lipids comprise a broad group of substances that have some common properties and compositional similarities (3). Triacylglycerols are fats and oils that represent the most prevalent category of the group of compounds known as lipids. The terms lipids, fats, and oils are often used interchangeably. The term "lipid" commonly refers to the broad, total collection of food molecules that meet the definition previously stated. Fats generally refer to those lipids that are solid at room temperature and oils generally refer to those lipids that are liquid at room temperature.

8.1.2 General Classification

The general classification of lipids that follows is useful to differentiate lipids in foods (3).

8.1.2.1 Simple Lipids

Ester of fatty acids with alcohol:

- **Fats**: Esters of fatty acids with glycerol—triacylglycerols.
- **Waxes**: Esters of fatty acids with long-chain alcohols other than glycerols (e.g., myricyl palmitate, cetyl palmitate, Vitamin A esters, and Vitamin D esters).

8.1.2.2 Compound Lipids

Compounds containing groups in addition to an ester of a fatty acid with an alcohol:

- **Phospholipids**: Glycerol esters of fatty acids, phosphoric acids, and other groups containing nitrogen (e.g., phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol).
- **Cerebrosides**: Compounds containing fatty acids, a carbohydrate, and a nitrogen moiety (e.g., galactocerebroside and glucocerebroside).
- **Sphingolipids**: Compounds containing fatty acids, a nitrogen moiety, and phosphoryl group (e.g., sphingomyelins).

8.1.2.3 Derived Lipids

Derived lipids are substances derived from neutral lipids or compound lipids. They have the general properties of lipids—examples are fatty acids, long-chain alcohols, sterols, fat-soluble vitamins, and hydrocarbons.

8.1.3 Content of Lipids in Foods

Foods may contain any or all types of the lipid compounds previously mentioned. The lipid content in bovine milk (Table 8-1) illustrates the complexity and variability of lipids in a food system, having lipids that differ in polarity and concentrations.

Foods contain many types of lipids, but those which tend to be of greatest importance are the triacylglycerols and the phospholipids. **Liquid triacylglycerols** at room temperature are referred to as **oils**, such as soybean oil and olive oil, and are generally of plant origin. **Solid triacylglycerols** at room temperature are termed **fats**. Lard and tallow are examples of fats, which are generally from animals. The term *fat* is applicable to all triacylglycerols whether they are normally solid or liquid at ambient temperatures. Table 8-2 shows the wide range of lipid content in different foods.

8-1 table **Lipids of Bovine Milk**

Kinds of Lipids	*Percent of Total Lipids*
Triacylglycerols	97–99
Diacylglycerols	0.28–0.59
Monoacylglycerols	0.016–0.038
Phospholipids	0.2–1.0
Sterols	0.25–0.40
Squalene	Trace
Free fatty acids	0.10–0.44
Waxes	Trace
Vitamin A	(7–8.5 μg/g)
Carotenoids	(8–10 μg/g)
Vitamin D	Trace
Vitamin E	(2–5 μg/g)
Vitamin K	Trace

Adapted from (4) with permission of S. Patton, and (5) copyright ©1959, John Wiley & Sons, Inc, with permission.

8-2 table **Fat Content of Selected Foods**

Food Item	*Percent Fat (Wet Weight Basis)*
Cereals, bread, and pasta	
Rice, white, long-grain, regular, raw, enriched	0.7
Sorghum	3.3
Wheat, soft white	2.0
Rye	2.5
Wheat germ, crude	9.7
Rye bread	3.3
Cracked-wheat bread	3.9
Macaroni, dry, enriched	1.6
Dairy products	
Milk, whole, fluid	3.3
Skim milk, fluid	0.2
Cheddar cheese	33.1
Yogurt, plain, whole milk	3.2
Fats and oils	
Lard, shortening, oils	100.0
Butter, with salt	81.1
Margarine, regular, hard, soybean	80.5
Salad dressing	
Italian, commercial, regular	48.3
Thousand Island, commercial, regular	35.7
French, commercial, regular	41.0
Mayonnaise, soybean oil, with salt	79.4
Fruits and vegetables	
Apples, raw, with skin	0.4
Oranges, raw, all commercial varieties	0.1
Blackberries, raw	0.4
Avocados, raw, all commercial varieties	15.3
Asparagus, raw	0.2
Lima beans, immature seeds, raw	0.9
Sweet corn, yellow, raw	1.2
Legumes	
Soybeans, mature seeds, raw	19.9
Black beans, mature seed, raw	1.4
Meat, poultry, and fish	
Beef, flank, separable lean and fat	10.6
Chicken, broilers or fryers, breast meat only	1.2
Bacon, pork, cured	57.5
Pork, fresh, loin, whole	12.6
Finfish, halibut, Atlantic and Pacific, raw	2.3
Finfish, cod, Atlantic, raw	0.7
Nuts	
Coconut meat, raw	33.5
Almonds, dried, unblanched, dry roasted	52.8
Walnuts, black, dried	56.6
Egg, whole, raw, fresh	10.0

From USDA Nutrient Database for Standard Reference, Release 14 (July 2001) http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl

8.1.4 Importance of Analysis

An accurate and precise quantitative and qualitative analysis of lipids in foods is important for accurate nutritional labeling, determination of whether the food meets the standard of identity, and to ensure that the product meets manufacturing specifications. Inaccuracies in analysis may prove costly for manufacturers and could result in a product of undesirable quality and functionality.

8.2 GENERAL CONSIDERATIONS

By definition, lipids are soluble in organic solvents and insoluble in water. Therefore, water insolubility is the essential analytical property used as the basis for the separation of lipids from proteins, water, and carbohydrates in foods. Glycolipids are soluble in alcohols and have a low solubility in hexane. In contrast, triacylglycerols are soluble in hexane and petroleum ether, which are nonpolar solvents. The wide range of relative hydrophobicity of different lipids makes the selection of a single universal solvent impossible for lipid extraction of foods. Some lipids in foods are components of complex lipoproteins and liposaccharides; therefore, successful extraction requires that bonds between lipids and proteins or carbohydrates be broken so that the lipids can be freed and solubilized in the extracting organic solvents.

8.3 ANALYTICAL METHODS

The total lipid content of a food is commonly determined by organic solvent extraction methods. The accuracy of these methods greatly depends on the solubility of the lipids in the solvent used and the ability to separate the lipids from complexes with other macromolecules. The lipid content of a food determined by extraction with one solvent may be quite different from the content determined with another solvent of different polarity. In addition to solvent extraction methods, there are nonsolvent wet extraction methods and several instrumental methods that utilize the physical and chemical properties of lipids in foods for fat content determination.

Many of the methods cited in this chapter are official methods of AOAC International. Refer to these methods and other original references cited for detailed instructions of procedures.

8.3.1 Solvent Extraction Methods

8.3.1.1 Sample Preparation

The validity of the fat analysis of a food depends on **proper sampling** and **preservation** of the sample before the analysis (see also Chapter 5). An ideal sample should be as close as possible in all of its intrinsic properties to the material from which it is taken. However, a sample is considered satisfactory if the properties under

investigation correspond to those of the bulk material within the limits of the test (7).

The sample preparation for lipid analysis depends on the type of food and type and nature of lipids in the food (8). The extraction method for lipids in liquid milk is generally different from that for lipids in solid soybeans. To analyze the lipids in foods effectively, knowledge of the structure, the chemistry, and the occurrence of the principal lipid classes and their constituents is necessary. Therefore, there is no single standard method for the extraction of all kinds of lipids in different foods. For the best results, sample preparation should be carried out under an inert atmosphere of nitrogen at low temperature to minimize chemical reactions such as lipid oxidation.

Several preparatory steps are common in lipid analysis, which act to aid in extraction by removal of water, reduction of particle size, or separation of the lipid from bound proteins and/or carbohydrates.

8.3.1.1.1 Predrying Sample Lipids cannot be effectively extracted with ethyl ether from moist food because the solvent cannot easily penetrate the moist food tissues due to the hydrophobicity of the solvents used or the hydroscopic nature of the solvents. The ether, which is hygroscopic, becomes saturated with water and inefficient for lipid extraction. Drying the sample at elevated temperatures is undesirable because some lipids become bound to proteins and carbohydrates, and bound lipids are not easily extracted with organic solvents. Vacuum oven drying at low temperature or lyophilization increases the surface area of the sample for better lipid extraction. Predrying makes the sample easier to grind for better extraction, breaks fat–water emulsions to make fat dissolve easily in the organic solvent, and helps to free fat from the tissues of foods (7).

8.3.1.1.2 Particle Size Reduction The extraction efficiency of lipids from dried foods depends on particle size; therefore, adequate grinding is very important. The classical method of determining fat in oilseeds involves the extraction of the ground seeds with selected solvent after repeated grinding at low temperature to minimize lipid oxidation. For better extraction, the sample and solvent are mixed in a high-speed comminuting device such as a blender. It can be difficult to extract lipids from whole soybeans because of the limited porosity of the soybean hull and its sensitivity to dehydrating agents. The lipid extraction from soybeans is easily accomplished if the beans are broken mechanically by grinding.

8.3.1.1.3 Acid Hydrolysis A significant portion of the lipids in foods such as dairy, bread, flour, and animal products is bound to proteins and carbohydrates, and direct extraction with nonpolar solvents is inefficient. Such foods must be prepared for lipid extraction by acid hydrolysis. Table 8-3 shows the inaccuracy that can occur if samples are not prepared by acid hydrolysis. Acid hydrolysis can break both covalently and ionically bound lipids into easily extractable lipid forms. The sample can be predigested by refluxing for 1 hr with 3 *N* hydrochloric acid. Ethanol and solid hexametaphosphate may be added to facilitate separation of lipids from other components before food lipids are extracted with solvents (6, 7). For example, the acid hydrolysis of two eggs requires 10 ml of HCl and heating in a water bath at 65°C for 15–25 min or until the solution is clear (6).

8-3 table **Effects of Acid Digestion on Fat Extraction from Foods**

	Percent Fat Acid Hydrolysis	*Percent Fat No Acid Hydrolysis*
Dried egg	42.39	36.74
Yeast	6.35	3.74
Flour	1.73	1.20
Noodles	3.77–4.84	2.1–3.91
Semolina	1.86–1.93	1.1–1.37

Adapted from (6), p. 154, with permission.

8.3.1.2 Solvent Selection

Ideal solvents for fat extraction should have a high solvent power for lipids and low or no solvent power for proteins, amino acids, and carbohydrates. They should evaporate readily and leave no residue, have a relatively low boiling point, and be nonflammable and nontoxic in both liquid and vapor states. The ideal solvent should penetrate sample particles readily, be in single component form to avoid fractionation, and be inexpensive and nonhygroscopic (6, 7). It is difficult to find an ideal fat solvent to meet all of these requirements. Ethyl ether and petroleum ether are the most commonly used solvents, but pentane and hexane are used to extract oil from soybeans.

Ethyl ether has a boiling point of 34.6°C and is a better solvent for fat than petroleum ether. It is generally expensive compared to other solvents, has a greater danger of explosion and fire hazards, is hygroscopic, and forms peroxides (6). **Petroleum ether** is the low boiling point fraction of petroleum and is composed mainly of pentane and hexane. It has a boiling point of 35–38°C and is more hydrophobic than ethyl ether. It is selective for more hydrophobic lipids, cheaper, less hygroscopic, and less flammable than ethyl ether. The detailed properties of petroleum ether for fat extraction are described in AOAC Method 945.16 (8).

A combination of two or three solvents is frequently used. The solvents should be purified and peroxide free and the proper solvent-to-solute ratio must be used to obtain the best extraction of lipids from foods (7).

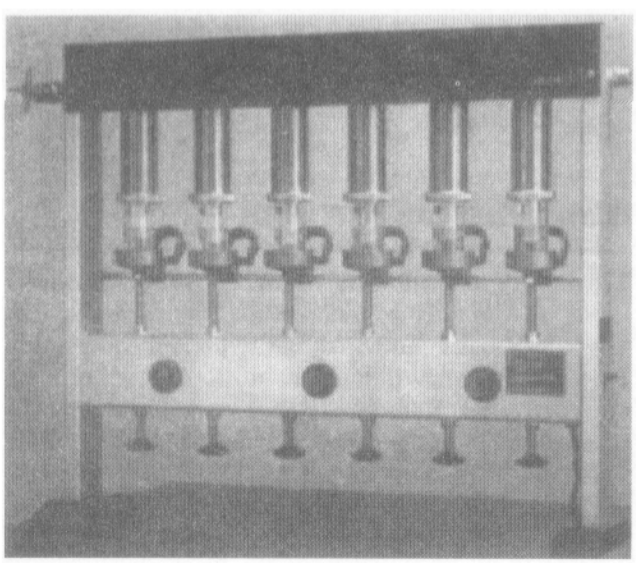
8.3.1.3 Continuous Solvent Extraction Method: Goldfish Method

8.3.1.3.1 Principle and Characteristics For continuous solvent extraction, solvent from a boiling flask continuously flows over the sample held in a ceramic thimble. Fat content is measured by weight loss of the sample or by weight of fat removed.

The continuous methods give faster, more efficient extraction than semicontinuous extraction methods. However, they may cause channeling that results in incomplete extraction. The Goldfish (as well as the Wiley and Underwriters) tests are examples of continuous lipid extraction methods (6, 7).

8.3.1.3.2 Procedure (See Fig. 8-1.)

1. Weigh predried porous ceramic extraction thimble. Place vacuum oven dried sample in thimble and weigh again. (Sample could instead be combined with sand in thimble and then dried.)
2. Weigh predried extraction beaker.
3. Place ceramic extraction thimble into glass holding tube and then up into condenser of apparatus.
4. Place anhydrous ethyl ether (or petroleum ether) in extraction beaker and put beaker on heater of apparatus.
5. Extract for 4 hr.
6. Lower heater and let sample cool.
7. Remove the extraction beaker and let air dry overnight, then at 100°C for 30 min. Cool beaker in desiccator and weigh.



8-1 figure Goldfish fat extractor. (Courtesy of Labconco Corp., Kansas City, MO.)

8.3.1.3.3 Calculations

$$\text{Weight of fat in sample} = (\text{beaker} + \text{fat}) - \text{beaker} \quad [1]$$

$$\%\ \text{Fat on dry weight basis} = (\text{g of fat in sample/g of dried sample}) \times 100 \quad [2]$$

8.3.1.4 Semicontinuous Solvent Extraction Method: Soxhlet Method

The Soxhlet method (AOAC Method 920.39C for Cereal Fat; AOAC Method 960.39 for Meat Fat) is an example of the semicontinuous extraction method and is described below.

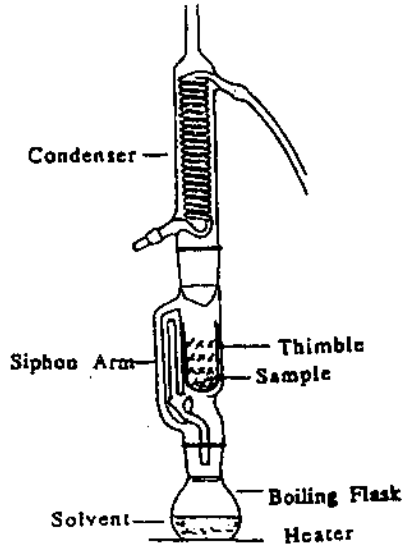
8.3.1.4.1 Principle and Characteristics For semicontinuous solvent extraction, the solvent builds up in the extraction chamber for 5–10 min and completely surrounds the sample, then siphons back to the boiling flask. Fat content is measured by weight loss of the sample or by weight of fat removed.

This method provides a soaking effect of the sample and does not cause channeling. However, this method requires more time than the continuous method. It should be noted that the Soxhlet method is often considered the standard methods by which others are evaluated.

8.3.1.4.2 Preparation of Sample If the sample contains more than 10% H_2O, dry the sample to constant weight at 95–100°C under pressure ≤100 mmHg for about 5 hr (AOAC Method 934.01).

8.3.1.4.3 Procedure (See Fig. 8-2.)

1. Weigh, to the nearest mg, about 2 g of predried sample into a predried extraction thimble, with porosity permitting a rapid flow of ethyl ether. Cover sample in thimble with glass wool.
2. Weigh predried boiling flask.
3. Put anhydrous ether in boiling flask. *Note*: The anhydrous ether is prepared by washing commercial ethyl ether with two or three portions of H_2O, adding NaOH or KOH, and letting stand until most of H_2O is absorbed from the ether. Add small pieces of metallic Na and let hydrogen evolution cease (AOAC Method 920.39B). Petroleum ether may be used instead of anhydrous ether (AOAC Method 960.39).



Soxhlet extraction apparatus.

8-2 figure Soxhlet extraction apparatus.

4. Assemble boiling flask, Soxhlet flask, and condenser.
5. Extract in a Soxhlet extractor at a rate of 5 or 6 drops per second condensation for about 4 hr, or for 16 hr at a rate of 2 or 3 drops per second by heating solvent in boiling flask.
6. Dry boiling flask with extracted fat in an air oven at 100°C for 30 min, cool in desiccator, and weigh.

8.3.1.4.4 Calculation

% Fat on dry weight basis

= (g of fat in sample/g of dried sample) × 100 [3]

8.3.1.5 Discontinuous Solvent Extraction Method: Mojonnier Method

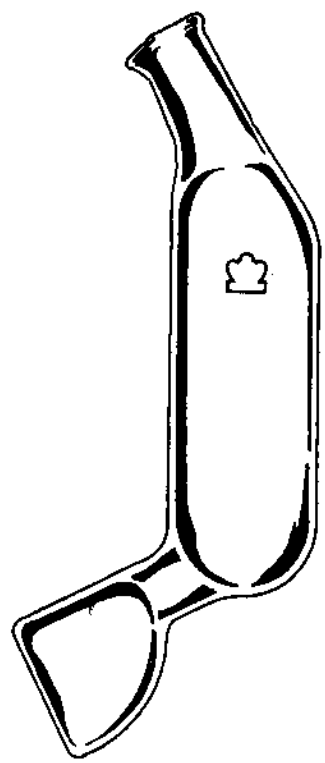
8.3.1.5.1 Milk Fat Method: Principle and Characteristics Fat is extracted with a mixture of ethyl ether and petroleum ether in a Mojonnier flask, and the extracted fat is dried to a constant weight and expressed as percent fat by weight.

The Mojonnier test is an example of the discontinuous solvent extraction method and does not require removal of moisture from the sample. It can be applied to both liquid and solid samples. It has been applied primarily to dairy foods, but is applicable to other foods. If petroleum ether is used to purify the extracted fat this method is very similar to the Roese–Gottlieb Method (AOAC Method 905.02) in both principle and practice.

8.3.1.5.2 Milk Fat Method (AOAC Method 989.05): Preparation of Sample Bring the sample to about 20°C; mix to prepare a homogeneous sample by pouring back and forth between clean beakers. Promptly weigh or measure the test portion. If lumps of cream do not disperse, warm the sample in a water bath to about 38°C and keep mixing until it is homogeneous, using a "policeman" if necessary to reincorporate the cream adhering to the container or stopper. When it can be done without interfering with dispersal of the fat, cool warmed samples to about 20°C before transferring the test portion.

8.3.1.5.3 Milk Fat Method: Procedure

1. Weigh, to the nearest 0.1 mg, 10 g of milk into a Mojonnier fat extraction flask (Fig. 8-3).
2. Add 1.5 ml of NH_4OH and shake vigorously. Add 2 ml if the sample is sour. NH_4OH neutralizes the acidic sample and dissolves protein.
3. Add 10 ml of 95% ethanol and shake for 90 sec. The alcohol prevents possible gel formation.
4. Add 25 ml of ethyl ether and shake for 90 sec. The ether dissolves the lipid.
5. Cool if necessary, and add 25 ml of petroleum ether and shake for 90 sec. The petroleum ether



8-3 figure Mojonnier fat extraction flask. (Courtesy of Kontes Glass Co., Vineland, NJ.)

Protein Analysis

Sam K. C. Chang

9.1 INTRODUCTION

9.1.1 Classification and General Considerations

Proteins are an abundant component in all cells, and almost all except storage proteins are important for biological functions and cell structure. Food proteins are very complex. Many have been purified and characterized. Proteins vary in molecular mass, ranging from approximately 5000 to more than a million daltons. They are composed of elements including hydrogen, carbon, nitrogen, oxygen, and sulfur. Twenty α-amino acids are the building blocks of proteins; the amino acid residues in a protein are linked by peptide bonds. Nitrogen is the most distinguishing element present in proteins. However, nitrogen content in various food proteins ranges from 13.4% to 19.1% (1) due to the variation in the specific amino acid composition of proteins. Generally, proteins rich in basic amino acids contain more nitrogen.

Proteins can be classified by their composition, structure, biological function, or solubility properties. For example, simple proteins contain only amino acids upon hydrolysis, but conjugated proteins also contain non-amino-acid components.

Proteins have unique conformations that could be altered by denaturants such as heat, acid, alkali, 8 *M* urea, 6 *M* guanidine-HCl, organic solvents, and detergents. The solubility as well as functional properties of proteins could be altered by denaturants.

The analysis of proteins is complicated by the fact that some food components possess similar physicochemical properties. Nonprotein nitrogen could come from free amino acids, small peptides, nucleic acids, phospholipids, amino sugars, porphyrin, and some vitamins, alkaloids, uric acid, urea, and ammonium ions. Therefore, the total organic nitrogen in foods would represent nitrogen primarily from proteins and to a lesser extent from all organic nitrogen-containing nonprotein substances. Depending upon methodology, other major food components, including lipids and carbohydrates, may interfere physically with analysis of food proteins.

Numerous methods have been developed to measure protein content. The basic principles of these methods include the determinations of nitrogen, peptide bonds, aromatic amino acids, dye-binding capacity, ultraviolet (UV) absorptivity of proteins, and light scattering properties. In addition to factors such as sensitivity, accuracy, precision, speed, and cost of analysis, what is actually being measured must be considered in the selection of an appropriate method for a particular application.

9.1.2 Importance of Analysis

Protein analysis is important for:

1. **Nutrition labeling.**
2. **Functional property investigation.** Proteins in various types of food have unique food functional properties: for example, gliadin and glutenins in wheat flour for breadmaking, casein in milk for coagulation into cheese products, and egg albumen for foaming. (See Chapter 15.)
3. **Biological activity determination.** Some proteins, including enzymes or enzyme inhibitors, are relevant to food science and nutrition: for instance, the proteolytic enzymes in the tenderization of meats, pectinases in the ripening of fruits, and trypsin inhibitors in legume seeds are proteins. To compare between samples, enzyme activity often is expressed in terms of specific activity, meaning units of enzyme activity per mg of protein.

Protein analysis is required when you want to know:

1. Total protein content
2. Content of a particular protein in a mixture
3. Protein content during isolation and purification of a protein
4. Nonprotein nitrogen
5. Amino acid composition (see Chapter 15)
6. Nutritive value of a protein (see Chapter 15)

9.1.3 Content in Foods

Protein content in food varies widely. Foods of animal origin and legumes are excellent sources of proteins. The protein contents of selected food items are listed in Table 9-1.

9.2 METHODS

Principles, general procedures, and applications are described below for various protein determination methods. Refer to the referenced methods for detailed instructions of the procedures. The Kjeldahl, Dumas (N combustion), and infrared spectroscopy methods cited are from the *Official Methods of Analysis* of AOAC International (3), and are used commonly in nutrition labeling and quality control. The other methods described are used commonly in research laboratories working on proteins. Many of the methods covered in this chapter are described in somewhat more detail in recent books on food proteins (4–6).

Table 9-1 Protein Content of Selected Foods

Food Item	Percent Protein (Wet Weight Basis)
Cereals and pasta	
Rice, brown, long-grain, raw	7.9
Rice, white, long-grain, regular, raw, enriched	7.1
Wheat flour, whole-grain	13.7
Corn flour, whole-grain, yellow	6.9
Spaghetti, dry, enriched	12.8
Cornstarch	0.3
Dairy products	
Milk, whole, fluid	3.3
Milk, skim, dry	36.2
Cheese, cheddar	24.9
Yogurt, plain, low fat	5.3
Fruits and vegetables	
Apple, raw, with skin	0.2
Asparagus, raw	2.3
Strawberries, raw	0.6
Lettuce, iceberg, raw	1.0
Potato, whole, flesh and skin	2.0
Legumes	
Soybeans, mature seeds, raw	36.5
Beans, kidney, all types, mature seeds, raw	23.6
Tofu, raw, firm	15.8
Tofu, raw, regular	8.1
Meats, poultry, fish	
Beef, chuck, arm pot roast	18.5
Beef, cured, dried beef	29.1
Chicken, broilers or fryers, breast meat only, raw	23.1
Ham, sliced, regular	17.6
Egg, raw, whole	12.5
Finfish, cod, Pacific, raw	17.9
Finfish, tuna, white, canned in oil, drained solids	26.5

From USDA Nutrient Database for Standard Reference, Release 14 (July 2001) http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl (2)

9.2.1 Kjeldahl Method

9.2.1.1 Principle

In the Kjeldahl procedure, proteins and other organic food components in a sample are digested with sulfuric acid in the presence of catalysts. The **total organic nitrogen** is converted to ammonium sulfate. The digest is neutralized with alkali and distilled into a boric acid solution. The borate anions formed are titrated with standardized acid, which is converted to nitrogen in the sample. The result of the analysis represents the crude protein content of the food since nitrogen also comes from nonprotein components.

9.2.1.2 Historical Background

9.2.1.2.1 Original Method In 1883, Johann Kjeldahl developed the basic process of today's Kjeldahl method to analyze organic nitrogen. General steps in the original method include:

1. **Digestion** with sulfuric acid, with the addition of powdered potassium permanganate to complete oxidation and conversion of nitrogen to ammonium sulfate.
2. **Neutralization** of the diluted digest, followed by **distillation** into a known volume of standard acid, which contains potassium iodide and iodate.
3. **Titration** of the liberated iodine with standard sodium thiosulfate.

9.2.1.2.2 Improvements Several important modifications have improved the original Kjeldahl process:

1. Metallic catalysts such as mercury, copper, and selenium are added to sulfuric acid for complete digestion. Mercury has been found to be the most satisfactory. Selenium dioxide and copper sulfate in the ratio of 3:1 have been reported to be effective for digestion. Copper and titanium dioxide also have been used as a mixed catalyst for digestion (AOAC Method 988.05) (3). The use of titanium dioxide and copper poses less **safety concern** than mercury in the post-analysis disposal of the waste.
2. Potassium sulfate is used to increase the boiling point of the sulfuric acid to accelerate digestion.
3. Sulfide or sodium thiosulfate are added to the diluted digest to help release nitrogen from mercury, which tends to bind ammonium.
4. The ammonia is distilled directly into a boric acid solution, followed by titration with standard acid.
5. Colorimetry Nesslerization, or ion chromatography to measure ammonia, is used to determine nitrogen content after digestion.

An excellent book to review the Kjeldahl method for total organic nitrogen was written by Bradstreet (7). The basic AOAC Kjeldahl procedure is Method 955.04. Semiautomation, automation, and modification for microgram nitrogen determination (micro Kjeldahl method) have been established by AOAC in Methods 976.06, 976.05, and 960.52, respectively.

9.2.1.3 General Procedures and Reactions

9.2.1.3.1 Sample Preparation Solid foods are ground to pass a 20-mesh screen. Samples for analysis should be homogeneous. No other special preparations are required.

9.2.1.3.2 Digestion Place sample (accurately weighed) in a Kjeldahl flask. Add acid and catalyst; digest until clear to get complete breakdown of

all organic matter. Nonvolatile ammonium sulfate is formed from the reaction of nitrogen and sulfuric acid.

$$\text{Protein} \xrightarrow[\text{Heat, catalyst}]{\text{Sulfuric acid}} (NH_4)_2SO_2 \qquad [1]$$

During digestion, protein nitrogen is liberated to form ammonium ions; sulfuric acid oxidizes organic matter and combines with ammonium formed; carbon and hydrogen elements are converted to carbon dioxide and water.

9.2.1.3.3 Neutralization and Distillation The digest is diluted with water. Alkali-containing sodium thiosulfate is added to neutralize the sulfuric acid. The ammonia formed is distilled into a boric acid solution containing the indicators methylene blue and methyl red (AOAC Method 991.20).

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3 + Na_2SO_4 + 2H_2O \qquad [2]$$

$$NH_3 + H_3BO_3 \text{ (boric acid)} \longrightarrow \underset{\text{(borate ion)}}{NH_4 + H_2BO_3} \qquad [3]$$

9.2.1.3.4 Titration Borate anion (proportional to the amount of nitrogen) is titrated with standardized HCl.

$$H_2BO_3^- + H^+ \longrightarrow H_3BO_3 \qquad [4]$$

9.2.1.3.5 Calculations

$$\text{Moles of HCl} = \text{moles } NH_3 = \text{moles N in the sample} \qquad [5]$$

A reagent blank should be run to subtract reagent nitrogen from the sample nitrogen.

$$\%N = N\,\text{HCl} \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\text{ g N}}{\text{mol}} \times 100 \qquad [6]$$

where:

N HCl = normality of HCl, in moles/1000 ml
Corrected acid vol. = (ml std acid for sample) −(ml std acid for blank)
14 = atomic weight of nitrogen

A factor is used to convert percent N to percent crude protein. Most proteins contain 16% N, so the conversion factor is 6.25 (100/16 = 6.25).

$$\%N/0.16 = \%\text{ protein}$$

or [7]

$$\%N \times 6.25 = \%\text{ protein}$$

Conversion factors for various foods are given in Table 9-2.

9-2 table **Nitrogen to Protein Conversion Factors for Various Foods**

	Factor
Egg or meat	6.25
Dairy products	6.38
Wheat	5.70
Other cereal grains and oilseeds	6.25
Almonds	5.18
Peanuts and Brazil nuts	5.46
Other tree nuts and coconut	5.30

Data from (3, 8).

9.2.1.3.6 Alternate Procedures In place of distillation and titration with acid, ammonia or nitrogen can be quantitated by:

1. Nesslerization:

$$4NH_4OH + 2HgI_2 + 4KI + 3KOH \xrightarrow[\text{mercuric iodide}]{} \underset{\text{ammonium dimercuric iodide, red-orange, 440 nm}}{NH_2Hg_2IO} + 7KI + 2H_2O \qquad [8]$$

This method is rapid and sensitive, but the ammonium dimercuric iodide is colloidal and color is not stable.

2. NH_3 + phenol + hypochlorite $\longrightarrow$ indophenol (blue, 630 nm) [9]
OH
3. pH measurement after distillation into known volume of boric acid.
4. Direct measurement of ammonia, using ion chromatographic method.

9.2.1.4 Applications

Advantages:

1. Applicable to all types of foods.
2. Inexpensive (if not using an automated system).
3. Accurate; an official method for crude protein content.
4. Has been modified (micro Kjeldahl method) to measure microgram quantities of proteins.

Disadvantages:

1. Measures total organic nitrogen, not just protein nitrogen.
2. Time consuming (at least 2 hr to complete).
3. Poorer precision than the biuret method.
4. Corrosive reagent.

9.2.2 Dumas (Nitrogen Combustion) Method

9.2.2.1 Principle

The combustion method was introduced in 1831 by Jean-Baptiste Dumas. It has been modified and automated to improve accuracy since that time. Samples are combusted at high temperatures (700–1000°C). The nitrogen released is quantitated by **gas chromatography** using a **thermal conductivity detector** (TCD) (9). The nitrogen determined is converted to protein content in the sample.

9.2.2.2 Procedure

Samples (approximately 100–500 mg) are weighed into a tin capsule and introduced to a combustion reactor in automated equipment. The nitrogen released is measured by a built-in gas chromatograph.

9.2.2.3 Applications

The combustion method is an alternative to the Kjeldahl method (10), and is suitable for all types of foods. AOAC Method 992.15 and Method 992.23 are for meat and cereal grains, respectively.

Advantages:

1. Requires no hazardous chemicals.
2. Can be accomplished in 3 min.
3. Recent automated instruments can analyze up to 150 samples without attention.

Disadvantages:

1. Expensive equipment is required.
2. Measures total organic nitrogen, not just protein nitrogen.

9.2.3 Infrared Spectroscopy

9.2.3.1 Principle

Infrared spectroscopy measures the **absorption of radiation** (near- or mid-infrared regions) by molecules in food or other substances. Different functional groups in a food absorb different frequencies of radiation. For proteins and peptides, various **mid-infrared** bands (6.47 μm) and **near-infrared** (NIR) bands (e.g., 3300–3500 nm, 2080–2220 nm, 1560–1670 nm) characteristic of the **peptide bond** can be used to estimate the protein content of a food. By irradiating a sample with a wavelength of infrared light specific for the constituent to be measured, it is possible to predict the concentration of that constituent by measuring the energy that is reflected or transmitted by the sample (which is inversely proportional to the energy absorbed) (11).

9.2.3.2 Procedure

See Chapter 24 for a detailed description of instrumentation, sample handling, and calibration and quantitation methodology.

9.2.3.3 Applications

Mid-infrared spectroscopy is used in Infrared Milk Analyzers to determine milk protein content, while NIR spectroscopy is applicable to a wide range of food products (e.g., grains, cereal, meat, and dairy products) (12, 13, 3) (AOAC Method 997.06). Instruments are expensive and they must be calibrated properly. However, samples can be analyzed rapidly (30 sec to 2 min) by analysts with minimal training.

9.2.4 Biuret Method

9.2.4.1 Principle

A violet-purplish color is produced when **cupric ions** are complexed with **peptide bonds** (substances containing at least two peptide bonds, that is, biuret, large peptides, and all proteins) under **alkaline conditions**. The absorbance of the color produced is read at 540 nm. The color intensity (absorbance) is proportional to the protein content of the sample (14).

9.2.4.2 Procedure

1. A 5-ml biuret reagent is mixed with a 1-ml portion of protein solution (1 to 10 mg protein/ml). The reagent includes copper sulfate, NaOH, and potassium sodium tartrate, which is used to stabilize the cupric ion in the alkaline solution.
2. After the reaction mix is allowed to stand at room temperature for 15 or 30 min, the absorbance is read at 540 nm against a reagent blank.
3. Filtration or centrifugation before reading absorbance is required if the reaction mixture is not clear.
4. A standard curve of concentration versus absorbance is constructed using **bovine serum albumin** (BSA).

9.2.4.3 Applications

The biuret method has been used to determine proteins in cereal (15, 16), meat (17), soybean proteins (18), and as a qualitative test for animal feed [AOAC Method 935.11 (refers to Methods 22.012–22.013, AOAC, 10th ed., 1965)] (19). The biuret method also can be used to measure the protein content of isolated proteins.

Advantages:

1. Less expensive than the Kjeldahl method; rapid (can be completed in less than 30 min); simplest method for analysis of proteins.
2. Color deviations are encountered less frequently than with Lowry, UV absorption, or turbidimetric methods (described below).
3. Very few substances other than proteins in foods interfere with the biuret reaction.
4. Does not detect nitrogen from nonpeptide or nonprotein sources.

Disadvantages:

1. Not very sensitive as compared to the Lowry method; requires at least 2–4 mg protein for assay.
2. Absorbance could be contributed from bile pigments if present.
3. High concentration of ammonium salts interferes with the reaction.
4. Color varies with different proteins; gelatin gives a pinkish-purple color.
5. Opalescence could occur in the final solution if high levels of lipid or carbohydrate are present.
6. Not an absolute method: color must be standardized against known protein (e.g., BSA) or against the Kjeldahl nitrogen method.

9.2.5 Lowry Method

9.2.5.1 Principle

The Lowry method (20, 21) combines the **biuret reaction** with the reduction of the **Folin–Ciocalteau phenol reagent** (phosphomolybdic-phosphotungstic acid) by **tyrosine** and **tryptophan** residues in the proteins. The bluish color developed is read at 750 nm (high sensitivity for low protein concentration) or 500 nm (low sensitivity for high protein concentration). The original procedure has been modified by Miller (22) and Hartree (23) to improve the linearity of the color response to protein concentration.

9.2.5.2 Procedure

The following procedure is based on the modified procedure of Hartree (23):

1. Proteins to be analyzed are diluted to an appropriate range (20–100 μg).
2. K Na Tartrate–Na_2CO_3 solution is added after cooling and incubated at room temperature for 10 min.
3. $CuSO_4$–K Na Tartrate–NaOH solution is added after cooling and incubated at room temperature for 10 min.
4. Freshly prepared Folin reagent is added, then the reaction mixture is mixed and incubated at 50°C for 10 min.
5. Absorbance is read at 650 nm.
6. A standard curve of BSA is carefully constructed for estimating protein concentration of the unknown.

9.2.5.3 Applications

Because of its simplicity and sensitivity, the Lowry method has been widely used in protein biochemistry. However, it has not been widely used to determine proteins in food systems without first extracting the proteins from the food mixture.

Advantages:

1. Very sensitive:
   a. 50–100 times more sensitive than biuret method;
   b. 10–20 times more sensitive than 280 nm UV absorption method (described below);
   c. Similar sensitivity as Nesslerization; however, more convenient than Nesslerization.
2. Less affected by turbidity of the sample.
3. More specific than most other methods.
4. Relatively simple; can be done in 1–1.5 hr.

Disadvantages:

For the following reasons, the Lowry procedure requires careful standardization for particular applications:

1. Color varies with different proteins to a greater extent than the biuret method.
2. Color is not strictly proportional to protein concentration.
3. The reaction is interfered with to varying degrees by sucrose, lipids, phosphate buffers, monosaccharides, and hexoamines.
4. High concentrations of reducing sugars, ammonium sulfate, and sulfhydryl compounds interfere with the reaction.

9.2.6 Bradford Dye-Binding Method

9.2.6.1 Principle

When **Coomassie Brilliant Blue G-250** binds to protein, the **dye changes color** from reddish to bluish, and the absorption maximum of the dye is shifted from 465 nm to 595 nm. The change in the absorbance at 595 nm is proportional to the protein concentration of the sample (24). Like other dye-binding methods, the Bradford relies on the **amphoteric nature of proteins**. When the protein-containing solution is acidified to a pH less than the isoelectric point of the protein(s) of

interest, the dye added binds electrostatically. Binding efficiency is enhanced by hydrophobic interaction of the dye molecule with the polypeptide backbone adjoining positively charged residues in the protein (4). In the case of the Bradford method, the dye bound to protein has a change in absorbance spectrum relative to the unbound dye.

9.2.6.2 Procedure

1. Coomassie Brilliant Blue G-250 is dissolved in 95% ethanol and acidified with 85% phosphoric acid.
2. Samples containing proteins (1–100 μg/ml) and standard BSA solutions are mixed with the Bradford reagent.
3. Absorbance at 595 nm is read against a reagent blank.
4. Protein concentration in the sample is estimated from the BSA standard curve.

9.2.6.3 Applications

The more rapid and sensitive Bradford method using Coomassie Brilliant Blue G-250 largely replaced other dye-binding methods that used anionic sulfonic acid dyes, including acid orange 12, orange G, and Amido black (25). The Bradford method has been used successfully to determine protein content in worts and beer products (26) and in potato tubers (27). This procedure has been improved to measure microgram quantities of proteins (28). Due to its rapidity, sensitivity, and fewer interferences than the Lowry method, the Bradford method has been used widely in protein purification.

Advantages:

1. Rapid; reaction can be completed in 2 min.
2. Reproducible.
3. Sensitive; several-fold more sensitive than the Lowry method.
4. No interference from ammonium sulfate, polyphenols, carbohydrates such as sucrose, or cations such as K^+, Na^+, and Mg^{+2}.
5. Measures protein or peptides with molecular mass approximately equal to or greater than 4000 Da.

Disadvantages:

1. Interfered with by both nonionic and ionic detergents, such as Triton X-100 and sodium dodecyl sulfate. However, errors due to small amounts (0.1%) of these detergents can be corrected using proper controls.
2. The protein-dye complex can bind to quartz cuvettes. The analyst must use glass or plastic cuvettes.
3. Color varies with different types of proteins. The standard protein must be selected carefully.

9.2.7 Bicinchoninic Acid (BCA) Method

9.2.7.1 Principle

Proteins reduce **cupric ions** to **cuprous ions** under **alkaline conditions** (29). The cuprous ion complexes with apple-greenish **BCA reagent** to form a purplish color. The color formed is proportional to protein concentration.

9.2.7.2 Procedure

1. Mix (one step) the protein solution with the BCA reagent, which contains BCA sodium salt, sodium carbonate, NaOH, and copper sulfate, pH 11.25.
2. Incubate at 37°C for 30 min, or room temperature for 2 hr, or 60°C for 30 min.
   The selection of the temperature depends upon sensitivity desired. A higher temperature gives a greater color response.
3. Read the solution at 562 nm against a reagent blank.
4. Construct a standard curve using BSA.

9.2.7.3 Applications

The BCA method has been used in protein isolation and purification. The suitability of this procedure for measuring protein in complex food systems has not been reported.

Advantages:

1. Sensitivity is comparable to that of the Lowry method; sensitivity of the micro BCA method (0.5–10 μg) is better than that of the Lowry method.
2. One-step mixing is easier than in the Lowry method.
3. The reagent is more stable than for the Lowry reagent.
4. Nonionic detergent and buffer salts do not interfere with the reaction.
5. Medium concentrations of denaturing reagents (4 *M* guanidine-HCl or 3 *M* urea) do not interfere.

Disadvantages:

1. Color is not stable with time. The analyst needs to carefully control the time for reading absorbance.
2. Any compound capable of reducing Cu^{+2} to Cu^+ will lead to color formation.

3. Reducing sugars interfere to a greater extent than in the Lowry method. High concentrations of ammonium sulfate also interfere.
4. Color variations among proteins are similar to those in the Lowry method.

9.2.8 Ultraviolet (UV) 280 nm Absorption Method

9.2.8.1 Principle

Proteins show strong absorption at **UV 280 nm**, primarily due to **tryptophan** and **tyrosine** residues in the proteins. Because the content of tryptophan and tyrosine in proteins from each food source is fairly constant, the absorbance at 280 nm could be used to estimate the concentration of proteins, using **Beer's law**. Since each protein has a unique aromatic amino acid composition, the extinction coefficient (E_{280}) or molar absorptivity (E_m) must be determined for individual proteins for protein content estimation.

9.2.8.2 Procedure

1. Proteins are solubilized in buffer or alkali.
2. Absorbance of protein solution is read at 280 nm against a reagent blank.
3. Protein concentration is calculated according to the equation

$$A = abc \qquad [10]$$

where:
A = absorbance
a = absorptivity
b = cell or cuvette path length
c = concentration

9.2.8.3 Applications

The UV 280 nm method has been used to determine the protein contents of milk (30) and meat products (31). It has not been used widely in food systems. This technique is better applied in a purified protein system or to proteins that have been extracted in alkali or denaturing agents such as 8 *M* urea. Although peptide bonds in proteins absorb more strongly at 190–220 nm than at 280 nm, the low UV region is more difficult to measure.

Advantages:

1. Rapid and relatively sensitive. (At 280 nm, 100 μg or more protein are required; several times more sensitive than the biuret method.)
2. No interference from ammonium sulfate and other buffer salts.
3. Nondestructive; samples can be used for other analyses after protein determination; used very widely in post-column detection of proteins.

Disadvantages:

1. Nucleic acids also absorb at 280 nm. The absorption 280 nm/260 nm ratios for pure protein and nucleic acids are 1.75 and 0.5, respectively. One can correct the absorption of nucleic acids at 280 nm if the ratio of the absorption of 280 nm/260 nm is known. Nucleic acids also can be corrected using a method based on the absorption difference between 235 nm and 280 nm (32).
2. Aromatic amino acid contents in the proteins from various food sources differ considerably.
3. The solution must be clear and colorless. Turbidity due to particulates in the solution will increase absorbance falsely.
4. A relatively pure system is required to use this method.

9.3 COMPARISON OF METHODS

- **Sample preparation:** The Kjeldahl, Dumas, and infrared spectroscopy methods require little preparation. Sample particle size of 20 mesh or smaller generally is satisfactory for these methods. Some of the newer NIR instruments can make measurements directly on whole grains and other coarsely granulated products without grinding or other sample preparation. Other methods described in this chapter require fine particles for extraction of proteins from the complex food systems.
- **Principle:** The Dumas and Kjeldahl methods measure directly the total amount of organic nitrogen element in the foods; other methods measure the various properties of proteins. For instance, the biuret method measures peptide bonds, and the Lowry method measures a combination of peptide bonds and the amino acids tryptophan and tyrosine. Infrared spectroscopy is an indirect method to estimate protein content, based on the energy absorbed when a sample is subjected to a wavelength of infrared radiation specific for the peptide bond.
- **Sensitivity:** Kjeldahl, Dumas, and biuret methods are less sensitive than Lowry, Bradford, BCA, or UV methods.
- **Speed:** After the instrument has been properly calibrated, infrared spectroscopy is likely the most rapid of the methods discussed. In most other methods involving spectrophotometric (colorimetric) measurements, one must separate proteins from the interfering insoluble materials before mixing with the color reagents

or must remove the insoluble materials from the colored protein-reagent complex after mixing. However, the speed of determination in the colorimetric methods and in the Dumas method is faster than with the Kjeldahl method.

9.4 SPECIAL CONSIDERATIONS

1. To select a particular method for a specific application, sensitivity, accuracy, and reproducibility as well as physicochemical properties of food materials must be considered. The data should be interpreted carefully to reflect what actually is being measured.

2. Food processing methods, such as heating, may reduce the extractability of proteins for analysis and cause an underestimation of the protein content measured by methods involving an extraction step (9).

3. All methods, except for the Dumas and Kjeldahl methods, and the UV method for purified proteins, require the use of a standard or reference protein or a calibration with the Kjeldahl method. In the methods using a standard protein, proteins in the samples are assumed to have similar composition and behavior compared to the standard protein. The selection of an appropriate standard for a specific type of food is important.

4. **Nonprotein nitrogen** is present in practically all foods. To determine **protein nitrogen**, the samples usually are extracted under alkaline conditions then precipitated with trichloroacetic acid or sulfosalicylic acid. The concentration of the acid used affects the precipitation yield. Therefore, nonprotein nitrogen content may vary with the type and concentration of the reagent used. Heating could be used to aid protein precipitation by acid, alcohol, or other organic solvents. In addition to acid precipitation methods used for nonprotein nitrogen determination, less empirical methods such as dialysis and ultrafiltration and column chromatography could be used to separate proteins from small nonprotein substances.

5. In the determination of the nutritive value of food proteins, including **protein digestibility** and **protein efficiency ratio** (PER), the Kjeldahl method with a 6.25 conversion factor usually is used to determine crude protein content. The PER could be underestimated if a substantial amount of nonprotein nitrogen is present in foods. A food sample with a higher nonprotein nitrogen content (particularly if the nonprotein nitrogen does not have many amino acids or small peptides) may have a lower PER than a food sample containing similar protein structure/composition and yet with a lower amount of nonprotein nitrogen.

9.5 SUMMARY

Methods based on the unique characteristics of proteins and amino acids have been described to determine the protein content of foods. The Kjeldahl and Dumas methods measure nitrogen. Infrared spectroscopy is based on absorption of a wavelength of infrared radiation specific for the peptide bond. Copper-peptide bond interactions contribute to the analysis by the biuret and Lowry methods. Amino acids are involved in the Lowry, dye-binding, and UV 280 nm methods. The BCA method utilizes the reducing power of proteins in an alkaline solution. The various methods differ in their speed and sensitivity.

In addition to the commonly used methods discussed, there are other methods available for protein quantification. Because of the complex nature of various food systems, problems may be encountered to different degrees in protein analysis by available methods. Rapid methods may be suitable for quality control purposes, while a sensitive method is required for work with a minute amount of protein. Indirect colorimetric methods usually require the use of a carefully selected protein standard or a calibration with an official method (e.g., Kjeldahl).

9.6 STUDY QUESTIONS

1. What factors should one consider when choosing a method for protein determination?
2. The Kjeldahl method of protein analysis consists of three major steps. List these steps in the order they are done, and describe in words what occurs in each step.
   Make it clear why milliliters of HCl can be used as an indirect measure of the protein content of a sample.
3. Why is the conversion factor from Kjeldahl nitrogen to protein different for various foods, and how is the factor of 6.25 obtained?
4. How can Nesslerization or the procedure that uses phenol and hypochlorite be used as part of the Kjeldahl procedure, and why might they best be put to use?
5. Differentiate and explain the chemical basis of the following techniques that can be used to quantitate proteins in quality control/research:
   a. Kjeldahl method
   b. Dumas method (N combustion)
   c. infrared spectroscopy
   d. biuret method
   e. Lowry method
   f. Bradford method
   g. BCA method
   h. absorbance at 280 nm
   i. absorbance at 220 nm
6. For each of the situations described below, identify a protein assay method most appropriate for use, and indicate

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Dietary Fiber

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I. INTRODUCTION

In the first edition of *Handbook of Food Analysis* the chapter on dietary fiber provided a comprehensive summary of over 40 methods, creating a record of the development of fiber analysis (1). In this revised chapter the focus is much more on methods currently in use and those appropriate for the analysis of human foods. The reader may refer to the first edition for details of the methods that are of particular interest.

Since the first edition there has been continued debate on the definition of dietary fiber and, linked to definition, the most appropriate methods for fiber analysis (2,3). A real attempt has been made to arrive at a single definition and method of analysis through international consultation. However, universal acceptance has not been reached. At least for regulatory and food labeling purposes, for which most dietary fiber analysis is required, there is, for the present time, a general acceptance of certified AOAC (Association of Official Analytical Chemists) International methods.

In this chapter, as the focus is on methods that are suitable for analyzing human foods, methods that were discussed in the first edition but are regarded as more suitable for ruminant feeds than for human foods, such as the neutral detergent method (4), will not be discussed.

Of recent reviews of the current state of dietary fiber analysis, that of Cho et al. gives detailed instructions on the AOAC methods (5). Brief overviews have been given by Asp (6) and McLeary (7). The report recently released by the Panel on the Definition of Dietary Fiber, Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Institute of Medicine (U.S.A.), gives an excellent overview (3).

II. DEFINING DIETARY FIBER

A. Dietary Fiber is Chemically Heterogeneous

The term "dietary fiber" was originally synonymous with "roughage"—the nondigestible plant matter, principally of cell wall origin, that is responsible for fecal bulking (8). Plant cell walls and their physiological impacts have, therefore, always been the core of the concept of dietary fiber, and they are the origin of most of the fiber in the great majority of natural foods—most fruits, vegetables, nuts, cereals, pulses, and their products. Indeed, the term dietary fiber was first explained by Hipsley as nondigestible constituents that make up the plant cell wall (9).

Whether defined as the nondigestible parts of plants, or the skeletal remains of the plant cell wall, dietary fiber is a portion of plant tissue with a particular property—digestion resistance in the human gut. It is, therefore, an array of compounds, rather than a unique chemical entity. Even when defined in its most chemically precise form, as nonstarch polysaccharides (NSPs), dietary fiber is heterogeneous. Plant cell wall

Table 1 Main Components That May Be Present in Dietary Fiber

Component	Characteristics
	Cell wall materials
Pectic substances	Heteropolysaccharides, predominantly polygalacturonic acid polymers and associated neutral fractions extracted from cell walls with hot water, hot Ca^{2+} chelating solutions (e.g., EDTA, ammonium oxalate), or dilute acid. About 35% of dicot primary wall. Usually a soluble fiber component
Hemicellulose	Complex polysaccharide mixture extracted from cell walls after pectin, with alkali (e.g., 10% KOH) for polymeric, or acid (e.g., 1.0 M H_2SO_4 100°C) for hydrolyzed. Major component of primary walls. Contributes mainly to insoluble fiber
Cellulose	Polysaccharide left after extracting pectic substances and hemicellulose. Main structural polymer in plants, high in fibrous tissues with secondary cell walls. Strong alkali insoluble, 72% (w/w) H_2SO_4 soluble at room temperature
Protein	About 10% of primary cell wall. Partly cross-linked with wall polysaccharides. Susceptible to proteases
Lignin	Left after 72% (w/w) H_2SO_4 cell wall extraction ("Klason lignin"). Extensive macromolecule from phenolic alcohols condensing in plant cell wall. Low in most foods
	Nondigestible carbohydrates not of cell wall origin
Gums and mucilages	Usually complex, heterogeneous polysaccharides of diverse origin—higher plant, algal, bacterial. Mainly nonstructural. Common features: water soluble, form viscous solutions or gels. Widely used in food industry, but small component of food. Higher plant: guar gum, gum arabic, gum tragacanth, larch gum. Algal: carrageenan, alginate. Bacterial: xanthan gum, whelan, gellan. Usually contribute to soluble fiber
Resistant starch	Amylase resistant. Mainly in starchy foods (cereals, pulses). Result of structure of food and/or starch granule impeding enzyme access to starch, or of starch retrogradation due to food processing. High amylose starches partly resistant. May add to insoluble fiber, depending on analysis method and fiber definition used. A small component of most foods
Man-made	For example, polydextrose, carboxymethylcellulose, lactulose
Storage polysaccharides	Storage polysaccharides analyzing as fiber include resistant starch, fructans, galactomannans
Chitin	Second most abundant natural polysaccharide. From invertebrate exoskeletons. Structure similar to cellulose but based on amino sugars. Not a common dietary fiber
	Associated substances
Cuticular substances	Complex waxes present on protective plant surfaces such as the epidermis. Contributes to insoluble fiber weight unless removed with organic solvent
Suberin	Long-chain fatty acid derivatives that occur in protective surfaces of the plant such as bark
Phenolic compounds	Polyhydroxyphenolic compounds such as tannins may condense or react with other food components to form compounds that analyze as lignin
Maillard products	Compounds that result from the reaction of sugars and proteins in heated foods. May analyze as lignin
Phytate	Inositol polyphosphate; associated with wheat bran and a range of legumes, but is not usually considered a true dietary fiber component. May impair mineral nutrition due to strong binding by polyphosphate groups

Source: Refs. 11–13.

polysaccharides in food remnants are polydisperse and polymolecular. They vary in their monosaccharide constituents, linkages, and chain lengths and, therefore, in their physiological properties (10).

In nature, and in the form of food residues that resist digestion in the small intestine, plant cell walls are coupled with a wide array of "associated substances." Substances that have been included in dietary fiber are shown in Table 1 (11–13). However, as a result of entrapment in plant tissues and food particles, almost any food component can reach the colon and exert an effect. Also, with expanding production of processed and functional foods in recent years there has been an enormous increase in intakes of noncell wall sources of NSPs added to foods as ingredients, and an increasing need for regulation of nutrient claims for dietary fiber. The concept of dietary fiber has, therefore, been extended to include

nondigestible polysaccharides, such as pectins, gums, mucilages, and resistant starch, and more recently oligosaccharides, such as oligofructans, which are not necessarily intrinsic components of plant cell walls (2,3).

B. Dietary Fiber is a Moving Target

Since the term dietary fiber was first proposed there has been constant discussion about the meaning of the term, and the definition has been revised several times. A brief summary of the definitions of dietary fiber to which most current methods for fiber analysis are linked is given in Table 2 (3).

The modern era of dietary fiber research was ushered in by Trowell (20) with the definition of dietary fiber as "the skeletal remains of the plant cell walls in foods which are resistant in the human digestive tract," and as "the portion of food which is derived from cellular walls of plants" (21). Therefore, although consisting principally of pectins, hemicelluloses, cellulose, and lignin, with associated cutin, suberin, and waxes, dietary fiber could, in theory, include a range of materials too numerous for practical or accurate analysis.

Trowell et al. (16) later defined dietary fiber more specifically as "the sum of the plant polysaccharides and lignin that are not digested by the endogenous secretions of the human gastrointestinal tract." This definition narrowed the concept of dietary fiber by focusing more specifically on nondigestible polysaccharides, but it broadened it to include plant polysaccharides not of cell wall origin, in response to increasing evidence for important roles for NSPs such as gums and mucilages in protecting against disease.

Trowell's definition of dietary fiber was almost equivalent to the "unavailable carbohydrate" component of foods, for which Southgate had developed methods to allow the correct calculation of the energy values of foods for British food tables (22). The work of Southgate, in the context of Trowell's dietary fiber definition, set in train a series of methodological

Table 2 Definitions of Dietary Fiber

Definition	Methods congruent with definition
Dietary fiber is NSP as measured by the Englyst method (15)	Chemical (NSP)
Dietary fiber consists of plant polysaccharides and lignin, which are resistant to hydrolysis by the digestive enzymes of man (16)	Chemical (NSP) plus gravimetric (lignin)
Dietary fiber is the endogenous components of plant materials in the diet that are resistant to digestion by enzymes produced by humans (17)	Gravimetric
Dietary fiber is the edible part of plant or animal material not hydrolyzed by endogenous enzymes of the human digestive tract as determined by the agreed upon method (18)	Gravimetric
Dietary fiber is material measured by AOAC method 985.29 or 991.43, and inulin and oligofructose (3)	Gravimetric plus limited supplementary methods for inulin and oligofructose
Dietary fiber is the edible parts of plants or analogous carbohydrate resistance to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine—includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fiber promotes beneficial physiological effects (19)	Gravimetric plus a potentially wide range of supplementary methods
Dietary fiber consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants. Added fiber consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans	Gravimetric plus a potentially wide range of supplementary specific methods
Total fiber is the sum of dietary fiber and added fiber (3). Any dietary component that reaches the colon without being absorbed in a healthy human gut (14). [Ha et al. (14) have proposed that dietary fiber no longer be regarded as a carbohydrate component of foods because many components that reach the colon with beneficial physiological effects are not carbohydrates]	An infinite range of specific methods

Source: Ref. 3.

developments in the UK, culminating in the measurement of dietary fiber specifically as NSPs (23), and the suggestion that the term "dietary fiber" be abolished (24). The methods developed involved chemical measurement of monosaccharide constituents, released by acid hydrolysis from material resistant to digestive enzymes after complete starch dispersion, and are therefore commonly referred to as the *enzymatic–chemical* methods.

Theander and coworkers (25) in Uppsala developed very similar methods to those of Englyst based on measurement of monosaccharide components of fiber, with the exception that they included enzyme-resistant starch, so measured nondigestible polysaccharides rather than NSPs, and included an enzymatic–gravimetric lignin component. Their *enzymatic–chemical–gravimetric* approach was, therefore, thought to be consistent with the definition of dietary fiber as nondigestible carbohydrate plus lignin.

While the specific chemical analysis of NSP was pursued in the U.K., in the U.S.A. method development aimed more at measuring dietary fiber gravimetrically as a conglomerate defined fiber as "... the polysaccharides and remnants of plant materials that are resistant to hydrolysis (digestion) by human alimentary enzymes" (5). The *enzymatic–gravimetric* approach was adopted as the basis of AOAC official methods (AOAC Methods 985.29 and 991.3) (5,26,27), in which dietary fiber is the weight of 80% ethanol-insoluble residue left after digesting with amylases and proteases, and allowing for protein and ash remaining in the residue.

Variants of the enzymic–gravimetric methods are the *nonenzymatic-gravimetric* method (28), in which enzymes are dispensed with altogether, and the *detergent–enzymatic/gravimetric* methods (29), which arose from the neutral detergent fiber analysis of Robertson and Van Soest (4).

1. Definitions and Methods Have Been Extended to 80% Ethanol-Soluble Carbohydrates

The enzymatic–chemical, enzymatic–gravimetric, and Uppsala methods all depend on an approximately 80% ethanol precipitation to recover dietary fiber that is soluble in the digestion medium of analysis. Such a step is, however, arbitrary, and does not always lead to complete precipitation of nondigestible carbohydrates. As the possible importance of 80% ethanol-soluble, nondigestible oligosaccharides to colonic health and energy balance has emerged, the need to include them through redefinition of dietary fiber, and prescription of additional methods, has arisen.

The American Association of Cereal Chemists recently attempted to obtain a consensus definition of dietary fiber, as an analytical/physiological entity, after extensive consultation and submissions from industry, academia, and government. The definition finally decided on was:

> Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fiber promotes beneficial physiological effects, including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation (19).

Such a definition, by including "edible parts of plants" and "associated substances" is clearly a move towards including any food components that are undigested in the definition of dietary fiber, including waxes, suberin, and cutin, so is a move away from the strictly NSP {[Englyst et al. (24)] and NSP plus lignin [Theander and Westerlund (25)]} definitions. A beneficial physiological effect is a prerequisite for inclusion. Because of the impracticality of measuring a large range of food components it is a definition tailored to the gravimetric methods with supplementary measurement of oligosaccharides where indicated.

The most recent concerted effort to define dietary fiber has been that of The Panel on the Definition of Dietary Fiber, a panel assembled by the Nutrition Board under the Standing Committee on the Evaluation of Dietary Reference Intakes, Institute of Medicine, in the U.S.A. (3). It has attempted to provide an accurate definition in which the role of dietary fiber in health is not specified, nondigestible plant polysaccharides and oligosaccharides are the core of the definition, and isolated, nondigestible carbohydrates added to food are permitted only if of demonstrable benefit. Their definition is:

- Dietary fiber consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants.
- Added fiber consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans.
- Total fiber is the sum of dietary fiber and added fiber.

This latest definition of dietary fiber, if adopted, will require further changes in methods to ensure that they provide a true measure of polysaccharides that are not digested under physiological conditions. In addition to monosaccharide composition and glycosidic bonds determining fiber content, as in most current methods, the complex influence of food structure in the gut will need to be accounted for. The proposed definition is likely to ignite a good deal of new debate.

III. ANALYZING DIETARY FIBER

A. Approaches to Dietary Fiber Analysis

A number of approaches have been taken to dietary fiber analysis, reflecting the lack of agreement on the definition of dietary fiber. The first methods were based on hot acid (30,31), acid detergent (32), or neutral detergent extraction (4) to determine the amount of non-nutritive matter in animal feeds. Such methods are not now considered appropriate for the analysis of human foods because much of the soluble NSP is lost in the discarded detergent extract.

Changes in definition have required changes in analytical methods, and in some cases the problem of matching method with definition has been solved pragmatically by defining dietary fiber as the material measured by a given method (3).

Current methods for measuring the dietary fiber in human foods fall into five main classes, summarised in Table 1. The methods that will be described within the classes are listed in Table 3.

1. Dimethyl Sulfoxide (DMSO)–Enzymatic–Chemical for NSP (33–36)

The enzymatic–chemical methods were devised for specifically measuring NSP. They use enzymatic digestion to remove starch and protein, and then measure remaining nondigestible polysaccharide specifically, as monosaccharide released by acid hydrolysis.

2. Enzymatic–Chemical/Gravimetric (25,37)

The enzymatic–chemical/gravimetric method removes digestible food components enzymatically, and measures nondigested polysaccharides including undigested starch, chemically, but includes a lignin residue determined gravimetrically, with allowance for protein and ash content.

3. Detergent–Gravimetric/Enzymatic–Gravimetric (29)

This approach attempts to overcome the loss of soluble dietary fiber in the detergent extract of detergent methods. Duplicate samples are extracted, one with hot neutral detergent to measure detergent fiber, and the other with buffer and amyloglucosidase to measure soluble fiber. The detergent fiber and soluble fiber are added to obtain a measure of total fiber.

4. Enzymatic–Gravimetric (26,27,38–43)

Enzymatic–gravimetric methods were developed for measuring polysaccharides, lignin, and associated substances that are resistant to digestion by the alimentary enzymes of humans. Enzymatic digestion is used to remove most starch and protein. Residual protein and ash content are deducted from the residue weight with the aim of obtaining a measure of NSP plus lignin by difference.

5. Nonenzymatic Gravimetric (28)

The nonenzymatic gravimetric procedure was developed to give a rapid measurement of dietary fiber in samples, such as ripe fruit, that contained little starch and protein, and enough fiber relative to starch to enable starch digestion to be dispensed with.

The principles underlying the various classes of method are summarized in Table 4.

B. Principles of Dietary Fiber Analysis

1. Dietary Fiber Analysis Depends on a Few Simple Principles

All approaches to dietary fiber analysis are based on attempts to meet four analytical requirements:

- The procedure should lead to the isolation of components resistant to human digestive enzymes, in accordance with the physiological conception of dietary fiber.
- All dietary fiber should be recovered from the digestion medium.
- Dietary fiber in the nondigestible food residue should be separated from other nondigestible components that do not fall within the operational definition of dietary fiber.
- The components constituting fiber should be accurately measured.

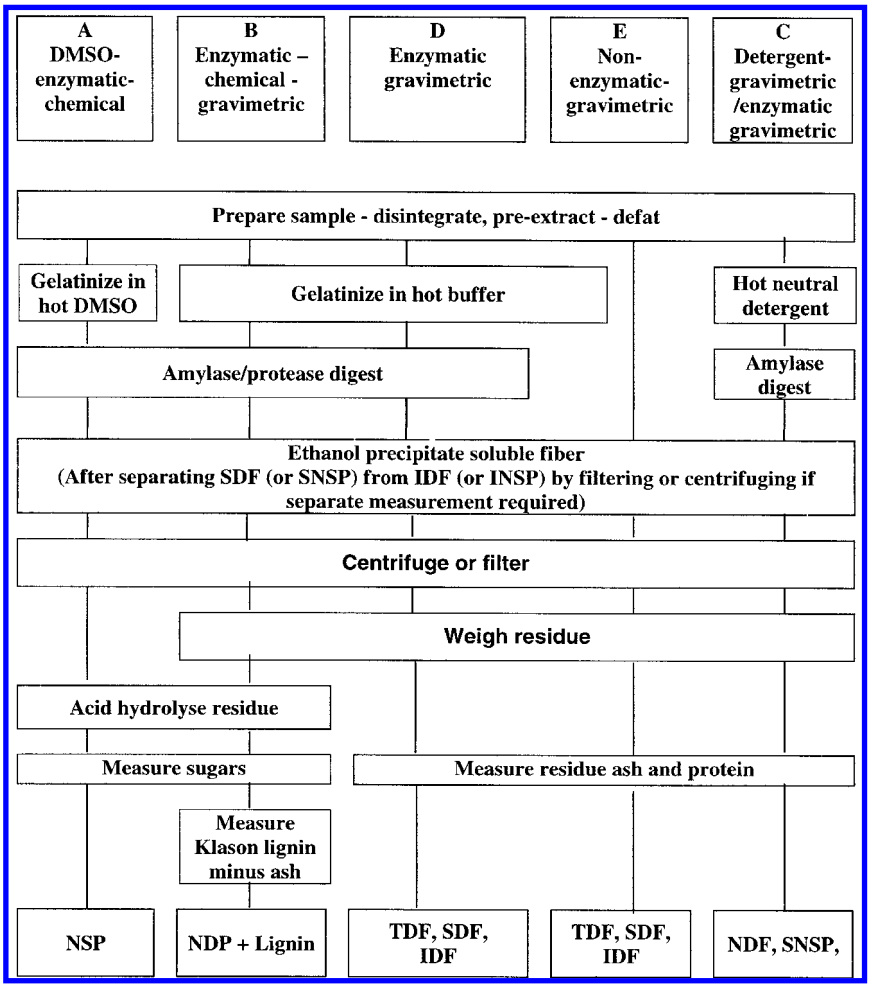

Figure 1 Main procedural differences between methods of dietary fiber analysis.

In addition, the need for tests to be practical enough to be carried out in most food laboratories has also been a guiding principle.

2. Analytical Goals are Seldom Reached in Fiber Analysis

In all methods for dietary fiber analysis, compromises that have been made for practical reasons and to minimize costs mean that analytical goals are seldom fully achieved. As a result, fiber analysis is usually not completely accurate.

- Preparation of samples for analysis, with defatting and grinding, means that physical constraints on digestion, such as particle size, will be quite different to those that would act in vivo. Also, the conditions used for digestion are seldom the same as those encountered by food in the digestive tract. Despite the intention of the methods using enzymes to provide a "physiological" measure of fiber as material resistant to the digestive enzymes of the human gut, methods have not been limited by a defined set of analytical conditions representing the gut lumen, in which the enzymes would normally act and that would determine the amount of fiber that would act in solution (44).
- In most methods, polysaccharide precipitation with 80% ethanol, a standard technique in polysaccharide chemistry, is used to recover soluble dietary fiber. However, use of 80% ethanol gives a somewhat arbitrary cut-off that does not always precipitate all nondigestible carbohydrates (45,46).
- Complete separation of nondigested carbohydrate from other nondigestible, nondietary

Table 3 Overview of Dietary Fiber Methods Detailed in This Chapter

Method	Sample digestion	Fiber measurement	Fraction measured	Reference
DMSO–enzymatic-chemical				
1. Faulks and Timms	DMSO–HS amylase—AGase	Acid hydrolysis, colorimetry	TNSP	33
2. Englyst and Hudson	DMSO–pancreatin–pullulanase	Acid hydrolysis, colorimetry	TNSP, INSP, SNSP by difference	34
3. Englyst et al.	DMSO–HS amylase–pancreatin–pullulanase	Acid hydrolysis, GLC, HPLC, or colorimetry	TNSP, INSP, by difference	35
4. Monro	Pepsin–pancreatin for PSNSP. DMSO–HS amylase–AGase for PINSP	Acid hydrolysis, GLC, HPLC, or colorimetry	TNSP, PINSP, PSNSP by difference	36
Enzymatic–chemical–gravimetric				
5. Uppsala	HS amylase–AGase	Acid hydrolysis, GLC, colorimetry, plus gravimetry	TNDP, INDP, SNDP, Klason lignin	25,37
Detergent–gravimetric/enzymatic–gravimetric				
6. Mongeau and Brassard	Detergent (NDF) + AGase (SDF)	Weigh, correct for ash and protein	NDF, SDF, TDF	29
Enzymatic–gravimetric				
7. Asp et al.	HS amylase–pancreatin–pepsin	Weigh, correct for ash and protein	TDF, IDF, SDF	38
8. Urea-Enz. Dial. I	HS amylase in 8 M urea then protease	Weigh, correct for ash and protein	TDF	39
9. Urea-Enz. Dial. II	HS amylase in 8 M urea then protease	Weigh, correct for ash and protein	IDF, SDF	40
10. Prosky TDF	HS amylase–AGase–protease phosphate buffer	Weigh, correct for ash and protein	TDF	41
11. Prosky SDF/IDF	HS amylase–AGase–protease phosphate buffer	Weigh, correct for ash and protein	IDF, SDF	26
12. Lee et al.	HS amylase–AGase–protease MES–tris buffer	Weigh, correct for ash and protein	TDF, IDF, SDF	27
13. Li and Andrews	AGase	Weigh, correct for ash and protein	TDF	42
14. Li and Cardozo I	AGase	Weigh, correct for ash and protein	IDF, SDF	43
Non-enzymatic-gravimetric				
15. Li and Cardozo II	No digestion	Weigh, correct for ash and protein	TDF	28

Abbreviations for all tables: AGase = amyloglucosidase; Col = colorimetric; Det = detergent; DMSO = dimethyl sulfoxide; Enz = enzymatic; EtOH = ethanol, GLC = gas–liquid chromatography; HS = heat stable; NDF = neutral detergent fiber; TNDP, INDP, SNDP = total, insoluble, and soluble nondigestible polysaccharide; NSP = nonstarch polysaccharide; RS = resistant starch; s/n = supernatant; TDF, SDF, IDF = total, insoluble, and soluble dietary fiber; temp = temperature; TNSP, SNSP, INSP = total, soluble, and insoluble NSP; PSNSP, PINSP = physiological SNSP and INSP; wt = weight.

fiber food components that could inflate dietary fiber values, whether by physical separation or by subtraction of analyzed protein and ash, is seldom achieved, particularly in gravimetric methods (47,48).

- Although method development has led to a high degree of reliability, accuracy is often questionable because analytical results may not specifically measure components described in the definition of fiber to which the method is

Table 4 Principles Underlying Major Approaches to Dietary Fiber Analysis Given in Table 3

1. *DMSO–enzymatic–chemical* (33–35)
   Principle: Ground sample is extracted with heat-stable amylase and a starch debranching enzyme (amyloglucosidase or pullulanase), after DMSO gelatinization of resistant starch. Soluble NSP is recovered by ethanol precipitation. Total NSP (containing ethanol-precipitated NSP) and insoluble NSP (soluble NSP is discarded) are measured as monosaccharides after acid hydrolysis and correction for hydrolysis losses. Soluble NSP is the difference between TNSP and INSP
   Comment: The SNSP does not represent that soluble under physiological conditions so its physiological relevance is questionable. The change in TNSP with and without physiological digestion before DMSO gives physiologically relevant NSP
2. *Enzymatic–chemical/gravimetric* (37)
   Principle: Heat-stable amylase–amyloglucosidase and protease is used to prepare a residue containing total nondigestible (includes resistant starch) polysaccharide, which is acid hydrolyzed for measurement as monosaccharide, with allowance for hydrolysis losses. The residue from acid hydrolysis is weighed and, after adjusting for ash content, is taken to be lignin. Total fiber is the sum of the nondigestible polysaccharide plus lignin
   Comment: In contrast to the DMSO–enzymatic–chemical approach, this approach measures a portion of the resistant starch present. However, it does not measure the true resistant starch load in vivo which depends on food structure destroyed during sample preparation for analysis. The enzymatic–chemical/gravimetric approach gives values that are similar to those from the enzymatic–gravimetric approach
3. *Detergent–gravimetric/enzymatic–gravimetric* (29)
   Principle: Sample is subjected to two distinct digestions. One involves detergent/amylase digestion to prepare a clean, low protein, insoluble fiber (IDF) preparation. The other involves extraction with hot water/amyloglucosidase, from which the soluble fiber (SDF) is recovered by ethanol precipitation. TDF is taken as the sum of IDF and SDF after allowing for the ash content of each
   Comment: A weakness is that TDF is the sum of IDF and SDF only if the IDF and SDF are prepared using the same extraction conditions, which is not the case with this approach
4. *Enzymatic–gravimetric* (26–28,38–43)
   Principle: Ground samples are enzymatically digested with protease and heat-stable amylase and/or amyloglucosidase, depending on method. Dissolved NSPs are ethanol-precipitated from the digest before filtering (for TDF), from the filtrate after filtering (for SDF), or from the retentate without ethanol precipitation (for IDF). Residues are dried and weighed and allowance is made for ash and protein contents using duplicate samples
   Comment: Extraction conditions mean that soluble fiber measured with this approach does not represent that soluble in the gut
5. *Nonenzymatic–gravimetric* (28)
   Principle: Sample is suspended in water, soluble polysaccharides are ethanol precipitated, and the insoluble residue is weighed and adjusted for protein and ash
   Comment: For use with foods containing very little starch and fat, and a relatively high proportion of dietary fiber, such as many fruits and vegetables. Removal of soluble sugars and adjustment for protein and ash in the residue gives a reasonable measure of TDF

linked. For instance, fiber measured as nondigestible polysaccharide plus lignin may be consistently overestimated due to the occurrence of materials that are not true lignins in the residue weighed as lignin, or underestimated in samples containing material of low molecular weight (47,48).

Definitions have, in some cases, been pragmatically modified to align them with analytical capabilities. For instance, the measurement of fiber as NSP arose from the impossibility of measuring "The remnants of plant cells resistant to the digestive enzymes of man" (20), which can, in theory, contain any plant component (49). NSP on the other hand, is amenable to precise chemical analysis. Gravimetric methods have not needed to be as specific in their associated definition.

C. Overview of Steps Common to Fiber Analysis Methods

1. Choosing a Method

If fiber analysis is to be carried out for the purposes of food labeling, methods will usually be prescribed by

food regulations, which should be checked to ensure that acceptable data are produced.

For the purposes of research, on the other hand, many methods have a place, but they should be selected for their ability to measure variables that are germane to the research. An appropriate operational definition of dietary fiber should be used, and a method congruent with the definition. A prerequisite to choosing a method is, therefore, a clear understanding, first, of relevant research variables and, second, of precisely what the method measures.

To understand the links between dietary fiber and health, certified dietary fiber methods such as those approved by AOAC are not necessarily appropriate. Definitions of dietary fiber, and associated methods, that rest on such general criteria as "nondigestible food carbohydrates" and "beneficial physiological effects in humans" may be too broad to be of use in nutrition research that tries to unambiguously identify links between specific nutritional factors and health because dietary fiber is a conglomerate of polymers with different properties.

Results may be more informative if a defined physiological property, as expressed under appropriate physiological conditions, is selected as an independent variable rather than the results of a certified fiber analysis conducted under the nonphysiological conditions typical of such methods. For instance, the solubility of dietary fiber under in vitro gastrointestinal conditions, and the increase in viscosity that it causes, would be more pertinent in exploring the links between soluble fiber and glycemic response to food than would soluble fiber measured by the Englyst or Prosky methods, neither of which use physiological gastrointestinal conditions. Lack of association between Englyst or AOAC soluble fiber and a physiological response may lead to the false conclusion that soluble fiber is not involved.

2. Preparing Samples

Preparing samples is an extremely important preliminary to most fiber determinations. Thorough extraction of nonfiber components, particularly available starch, is necessary to avoid overestimating fiber in the methods listed in Table 3. Samples must, therefore, be sufficiently fragmented and defatted for polymers to diffuse freely and/or for enzymes to gain unhindered access to their substrates during digestion.

However, the particle sizes of about 0.5 mm or less recommended for current certified methods prevent them from providing a realistic measure of the dietary fiber loading because the effects of food structure are destroyed in sample preparation. If the recent proposals of the Institute of Medicine, Panel on the Definition of Dietary Fiber (3), are adopted, dietary fiber methods for food labeling will be required to provide a more realistic measure of resistant starch. Then, methods that simulate chewing and gut conditions will be required. Several methods have already been devised for measuring total resistant starch in foods that have been chewed (50,51) or subjected to simulated chewing (52,53) rather than ground, so that usual structural impediments to starch digestion remain intact. Such methods can be easily coupled with measurement of gravimetric resistant starch, NSP, and lignin (54).

For methods that use grinding or milling to avoid the effects of food structure on dietary fiber analysis (all methods in Table 3), sample pretreatment should be the minimum necessary to facilitate sample digestion and fiber measurement because processes such as heating, dehydrating, and grinding may alter molecular interactions within and between dietary fiber and other food components (45,48). Changes induced secondarily to particle size reduction may reduce the validity of methods that aim to measure the potential amounts and properties (such as solubility) of fiber as consumed in foods, or the amount of material intrinsically resistant to digestion in food as consumed. Hot drying may lead to artifacts such as Maillard products and resistant starch (45,48), and is likely to cause partial degradation of the fiber, altering its solubility. Maillard products behave as lignin in fiber analysis, inflating fiber values from gravimetric methods that include lignin in fiber, particularly if there is no correction made for residual protein (55). Very small particle size has been associated with loss of fiber in analysis, possibly due to increased reactive surface or losses of fine material during filtration (47,56,57).

Dry milling or grinding, before or after defatting, is the most common means of disintegrating samples. A dry, homogeneous powder is convenient for accurately obtaining a representative subsample for analysis. Sample disruption would be achieved more physiologically with a short period of liquid homogenization or wet ball milling if the purpose of analysis is to measure dietary fiber components in food closer to the state in which they are consumed.

Neither milling nor homogenization will have an effect similar to chewing and are, therefore, not truly physiological. However, thorough tissue disruption is necessary to extract starch and protein successfully and, provided that physiological conditions are used,

allows a measure of the potential, if not actual, effect of the chemical environment of the gut on the solubility of food cell wall polysaccharides that is free from the variable effect of gross food structure.

Samples with a fat content greater than 5–10% are usually defatted. The fat level that can be tolerated without affecting fiber analysis has not been determined, but will depend on fat in concert with other properties of a food.

Defatting–depigmenting methods have used a range of solvents, including acetone, chloroform, chloroform–methanol, diethyl ether, ethanol, 90% ethanol, hexane, and petroleum spirits (1). Alcohol pretreatments should be avoided when soluble fiber is to be measured because ethanolic dehydration may lead to retrogradation of starch, and alter the solubility of some fiber polysaccharides (58).

3. Digesting samples

The main objective of sample digestion is to extract and/or degrade food components that are not part of dietary fiber as defined for a method, and that would invalidate fiber measurements if not removed. If the aim is to measure nondigestible polysaccharide it is most important to extract digestible starch because it will behave as apparent cell wall polysaccharide, contributing to fiber measured either gravimetrically or as polysaccharide sugars.

Protein is also digested in most methods, although a correction may be applied by subtracting residual protein as $N \times 6.25$. However, as it is an approximate correction, it could lead to error when the levels of residual protein are high.

a. Why Use Enzymes? Nondigestibility by enzymes secreted by the human gut is a central feature of all of the dietary fiber definitions outlined in Table 2, and, even if not stated, it is the implied basis of all of the methods in Table 3. Enzymes with similar digestive capacities to those in the mammalian gut are used to provide a measure of fiber that is consistent with its "physiological" definition as a nondigestible food component. However, although enzymic digestion is often related to the physiological concept of dietary fiber, use of enzymes introduces specificity rather than making a method physiological because human enzymes, and the conditions of the human gut, are not used in fiber analysis.

The specificity of enzymes is a valuable tool for the selective digestion of starch and protein from NSPs, allowing measurement of dietary fiber when NSPs or nondigestible carbohydrate are part of the definition.

The main enzymes used during digestion in dietary fiber analysis are amylases and amyloglucosidases (or pullulanase) from fungi or bacteria, to remove starch, and proteases from a variety of sources, including porcine (pepsin/pancreatin), bacterial, or fungal. If starch is not thoroughly extracted dietary fiber values will be spuriously high when NSP is to be measured, irrespective of the method used. Protein extraction is most important in gravimetric methods, although high protein concentrations may also interfere with colorimetry (59).

Amylase and amyloglucosidase are both *endo*-enzymes, but amylase has difficulty hydrolyzing α-(1–4) bonds in the vicinity of α-(1–6) branch points in starch, so that starch fragments (limit dextrins) are produced when amylase alone is used to digest starch. Any limit dextrins precipitated by 80% ethanol will contribute to fiber in methods using an 80% ethanol precipitation step to recover soluble fiber from the digestion/extraction medium. The problem is overcome by including amyloglucosidase or pullulanase to complete the hydrolysis of starch and protein.

Thermostable amylases have become a standard part of dietary fiber analysis because they provide the opportunity to achieve starch gelatinization and digestion concurrently. The universal practice of gelatinizing and digesting starch at the same time has been a double-edged sword, however. It results in all food samples being "cooked" at an early stage in analysis and, therefore, reduces the nutritional relevance of data from foods eaten raw, and obliterates the effects of heat processing on any fiber properties, such as solubility, that might be measured as part of the analysis (36).

b. Using Enzymes. Purity of starch-degrading enzymes is of the utmost importance in dietary fiber analysis to ensure that depolymerization of NSPs due to contaminating activities does not occur (60). Amylases are often produced by bacteria and fungi as part of a battery of digestive enzymes, and even on pure substrates small amounts of enzymes in addition to that specific to the substrate may be produced.

The purity of enzyme preparations should be pretested to ensure that they are not active against fiber under the conditions used, but have the desired activities, by running through the entire procedure with model substrates. For example, enzyme activities and substrates used to detect them, as used by the Prosky (26) and Lee et al. (27) AOAC methods, have been pectinase (citrus pectin), hemicellulase (strachtan = larch gum), and β-glucanase (-glucan), with

expected substrate recoveries of 95–100%, and amylase (wheat and corn starches) and protease (casein), with 1–2% expected recoveries. Substrates to test for evidence of contaminating activities may be provided as part of dietary fiber kits (Sigma and Megazyme).

The problem of enzyme contamination has now been largely overcome with the availability of electrophoretically pure enzymes developed especially for dietary fiber analysis (Megazyme Int. Ireland Ltd., Bray, Eire).

As well as specificity, the concentration and activity of enzymes need to be carefully controlled, particularly where fiber recovery depends on the extent to which resistant starch is degraded during analysis. Resistant starch is not absolutely amylase resistant, but results from retarded digestion so that a portion of very slowly available starch remains at the end of digestion. Differences in activity will determine the size of the resistant starch residue and, therefore, influence the amount of dietary fiber measured (60).

4. Fractionating Fiber

Many methods are designed to achieve some fractionation of dietary fiber into its components. Separation into soluble and complementary insoluble fiber fractions is most common, but is usually based on solubility under analytical conditions designed to maximize starch digestion rather than on solubility under the physiological conditions of the gastrointestinal tract.

Differences in digestion conditions vary from method to method, and lead to method-dependent differences in both distribution of polysaccharides between fractions, and in composition within the fractions (44,47,61–65). For instance, in raw fruit and vegetables the distribution of polysaccharides between soluble and insoluble fractions may be strongly governed by the effect of extraction conditions on pectic substances. Hot phosphate at pH 7 appears to extract Ca^{2+}, while heat at neutral pH will favor β-elimination; the combined sensitivity to buffer species and pH leads to disrupted junction zones and depolymerization, increasing the solubility of pectic polyuronide and associated polysaccharides compared with methods using hot acetate buffer at pH 5 or physiological conditions (44).

When DMSO is used to gelatinize starch prior to digestion, in methods measuring dietary fiber as NSP (Table 3), it may extract some otherwise insoluble cell wall polysaccharide (66,67), inflating SNSP (soluble NSP) values at the expense of INSP (insoluble NSP). This potential problem is overcome by extracting soluble fiber under physiological conditions, before DMSO treatment (36).

The Southgate (22) and early Englyst (23,68) methods base their approach to fiber fractionation on the traditional division of cell wall polysaccharides into the pectic substances, hemicellulose and cellulose. Such methods may provide more detail of fiber composition than other methods, particularly when coupled with GC analysis of the constituent sugars, but are considered exacting and too elaborate for routine use.

Recent discussions of dietary fiber analysis have recommended that the soluble–insoluble fiber classification is phased out (3) because the physicochemical properties, viscosity and fermentability, are more important to health than solubility per se. Of course, the same argument can be equally applied to dietary fiber—is it worth measuring when it is the effect of undigested food residues, rather than the amount of dietary fiber in the form of undigested carbohydrate and lignin, that is important in nutrition?

5. Measuring Fiber

Fiber is measured either by nonspecific gravimetry or by specific chemical methods for measuring the fiber polysaccharides as their monosaccharide constituents after hydrolysis, and by combinations of the two approaches (Table 3). The approved dietary fiber methods have been critically reviewed and described in more detail than is possible here (5).

a. Gravimetric Methods. Gravimetric methods measure fiber as the weight of residue remaining after digestion, usually corrected for protein and ash. Protein is calculated from total nitrogen measured by the Kjeldahl method in one of a duplicate set of samples, using the equation: protein = N × 6.25. Ash is usually determined by subtracting the weight of the residue holder (most often crucible plus Celite) from the holder plus ash. Protein and ash determinations are also conducted on blanks so that the corrected fiber content of the blank is obtained. Fiber in the sample can then be calculated:

$$\begin{aligned}\text{Sample fiber} = {} & \text{weight of digested residue} \\ & - \text{residue protein} - \text{residue ash} \\ & - \text{fiber in blank (adjusted for ash} \\ & \quad \text{and protein in blank)}\end{aligned}$$

The gravimetric methods for measuring fiber are relatively easy and precise. They do not require the sophisticated equipment or expertise demanded by

GLC analysis, nor the skill and control that seem necessary with some of the more sensitive colorimetric methods. However, the need for Kjedahl nitrogen and ash in order to make corrections to the final residue weight means that the methods are still quite labor intensive. Equipment for centrifuging or filtering, ovens for drying and ashing, an accurate balance, water baths, and a pH meter are the main requirements, and are available to most analytical laboratories.

Gravimetric methods are nonspecific in so far as they measure any residue components, including resistant starch, that survive the pre-extraction and digestion stages of analysis, are insoluble in 80% ethanol, and are not covered by the correction for protein and ash. Tannins and tanned food constituents, Maillard products, and other artifacts from food processing may be present and lead to overestimation of fiber (47,48,64,65), and of lignin when measured separately (55), particularly in samples (such as white bread) containing only small amounts of dietary fiber (48). The contribution of such nonspecific materials has been considered especially important in foods that are intrinsically unusual in containing, for instance, high levels of tannin (carob pods) or an unusual processing history.

Because of their nonspecificity, it may be difficult to assess the accuracy with which gravimetric methods determine dietary fiber—defined as nondigestible polysaccharide plus lignin—for a particular food. Where direct comparisons have been made between gravimetric and specific chemical methods, the gravimetric methods have often given higher values for dietary fiber than when a food is measured as specific food components such as NSP (48,49,65,69,70).

Correcting for residual protein using $N \times 6.25$ evidently corrects only partly for Maillard products, and the factor 6.25 is derived from a particular set of amino acids but is otherwise arbitrary. Therefore, it is best to aim for a low level of protein in fiber residues to avoid error associated with the correction by including a protease in the digestion sequence, as is the case in most dietary fiber methods.

b. Nongravimetric Methods. Nongravimetric methods for analyzing dietary fiber measure the sugar constituents of the fiber polysaccharides by GLC or HPLC, in combination with colorimetry (35,37) or by colorimetry alone (33,34). Acidic cell wall sugars (galacturonic and glucuronic acids) are difficult to measure under the same conditions as neutral sugars during GC and HPLC so are measured separately by colorimetry (35), although the Theander method recommends potentiometry as an alternative (37). The specific colorimetric methods that have been used in dietary fiber analysis are summarized in Table 5 (34,71–74). Southgate has provided detailed instructions for carrying out a range of colorimetric measurements on food polysaccharides (59), including methods, such as the anthrone and carbazole methods, which have been largely replaced but which are shown in Table 5 for completeness.

The application of chromatographic methods, widely used for polysaccharide analysis in carbohydrate chemistry, to the analysis of dietary fiber can be attributed primarily to Englyst et al. (35) and to Theander et al. (37) The two groups have developed similar GLC methods based on preparation of a digestion-resistant dietary fiber residue, acid hydrolysis (12 M H_2SO_4) of the residue to monosaccharides, alditol acetate derivatization of the hydrolyzate monosaccharides with an internal standard, and correction factors for hydrolysis losses. Uronic acids are determined separately, in both methods, by the Scott procedure (72) or by potentiometry. The main difference is that Englyst et al. pretreat the sample with hot DMSO to gelatinize all starch to a digestible dispersion, so that the procedure measures NSP. The Theander method includes starch that remains digestion resistant after grinding the sample, and also includes the residue after acid hydrolysis (Klason lignin) in the final calculation of dietary fiber.

GLC analysis allows a total fiber value to be calculated from the monosaccharide constituents, but provides further information on monosaccharide composition. While such detail is of interest in relation to the composition of polysaccharides that contribute to fiber, it is not usually required in the analysis of dietary fiber per se as a component in foods, especially for the purposes of food labeling.

With recent developments in high-performance liquid chromatography (HPLC) columns, and in the detection of separated sugars in column effluent by pulsed amperometric detection, HPLC is a viable alternative to GLC for measuring monosaccharides in fiber hydrolyzates (35,75). HPLC has the advantage over GLC of giving high-resolution separations and sensitive and specific detection of sugars without the need for derivatization. Furthermore, it is capable of specifically measuring a wide range of saccharides of different degrees of polymerization, so it is likely to play an important role in measuring nondigestible food carbohydrates that are not of cell wall origin, such as oligosaccharides.

Table 5 Colorimetric and Chromatographic Methods for Measuring Monosaccharides in Acid Hydrolyzates of Dietary Fiber Preparations in Enzymatic–Chemical Methods

Method	Reference
Colorimetric Anthrone reaction (hexoses) To 1 mL of test solution (25–200 μg sugar/mL) in a glass-stoppered tube add 10 mL anthrone reagent [10 g thiourea, 0.5 g anthrone/L 66% (v/v) H_2SO_4]. Equilibrate at room temp, heat 100°C, 15 min. Cool and read at 620 nm after 20–30 min	59
Orcinol/ferric chloride reaction (pentoses) To 3 mL test solution (2–10 μg pentose/mL) in a glass-stoppered tube add 3 mL orcinol/$FeCl_3$/HCl reagent [10% orcinol in EtOH : 0.1% (w/v) anhydrous $FeCl_3$ in concentrated HCl; 1:10]. Heat 100°C, 45 min. Cool and read at 670 nm	59
p-Hydroxybenzoic acid hydrazide (PAHBAH) reaction (reducing sugars) To 1 mL sample containing up to 16 μg/mL sugar add 2 mL 1% (w/v) PAHBAH solution (2% PAHBAH in 0.5 M HCl–1 mM $CaCl_2$: 3 M NaOH, 1:1). Heat 100°C, 5 min. Cool 15–20 s in ice–water, read at 410 nm	71
Dinitrosalicylate (DNS) reaction (reducing sugars) To 1 mL sugar solution (hydrolyzate) containing 0.5–2.0 mg/mL sugar add 0.5 mL glucose solution (0.5 mg/mL) and 0.5 mL 3.9 M NaOH. Mix. Add 2 mL DNS solution (10 g 3,5-dinitrosalicylic acid + 16 g NaOH + 300 g Na/K tartarate made to 1 L with water). Heat 100°C, 10 min. Cool. Add 20 mL water, mix. Read absorbance at 530 nm	34
Dimethylphenol (DMP) reaction for (uronic acids) To 0.3 mL of hydrolyzate (max. uronic acid 150 μg/mL, dilute with 2 M H_2SO_4) add 0.3 mL NaCl–H_3BO_3 (2 g NaCl plus 3 g H_3BO_3 in 100 mL water) and mix. Add 5 mL conc. H_2SO_4 and mix immediately. Heat 70°C, 40 min. Cool. Add 0.2 mL DMP solution (0.1 g 3,5-DMP in 100 mL glacial acetic acid). Read between 10 and 15 min later at 400 nm and 450 nm. Subtract 400 from 450 nm reading. Use 0.91 as factor for converting monosaccharides into polysaccharides	72
m-Hydroxydiphenyl (MHDP) reaction (uronic acids) To 1 mL solution containing 5–75 μg/mL uronic acids add 6 mL H_2SO_4/tetraborate solution (0.0125 M Na tetraborate in conc. $10H_2SO_4$) with tubes in ice. Immediately mix thoroughly. Heat 100°C, 5 min exactly and cool. Add 0.1 mL MHDP solution (0.15% MHDP in 0.5% NaOH). Immediately mix thoroughly. Include sample blanks replacing MHDP solution with 0.5% NaOH. After 15 min read at 520 nm. Subtract sample blank from reading	73,74
Carbazole reaction (uronic acids) Add 1 mL test solution containing 4–40 μg/mL uronic acid to 5 mL borate/H_2SO_4 (0.025 M Na tetraborate. $10H_2O$ in conc. H_2SO_4) at 4°C. Mix gently, heat at 100°C, 10 min. Cool to room temp, add 0.2 mL carbazole solution [0.125% (w/v) carbazole in EtOH], and mix. Read absorbance at 530 nm	59
Gas–liquid chromatography (GLC) (neutral sugars) Alditol acetate derivatization of sugars for GLC: To 3 mL hydrolyzate add 1 mL internal standard (1 mg allose/mL 50% saturated benzoic acid). With tubes in ice–water add 1 mL 12.5 M ammonia solution (may need a little extra) until alkaline. Add 5 μL octan-2-ol and 0.2 mL ammonia–$NaBH_4$ solution (1.2 g $NaBH_4$ in 6 mL 12.5 M ammonia solution). Mix and heat 40°C, 30 min. Add 0.4 mL glacial acetic acid and mix. Remove 0.5 mL to 30 mL glass tube and add to it 0.5 mL 1-methylimidazole, 5 mL acetic anhydride, and immediately mix. Leave 10 min, add 0.9 mL absolute EtOH, mix, and stand 5 min. Add 10 mL water, mix, and stand 5 min. Add 0.5 mL bromophenol blue solution (0.4 g/L). With tubes in ice–water add 5 mL 7.5 M KOH. After 2 min again add 5 mL 7.5 M KOH. Mix by inversion and after phase separation remove upper phase avoiding lower (blue) phase. Inject 0.5–1.0 μL for conventional GLC of the alditol acetate derivatives Englyst et al. (35) used calibration ratios based on previously determined recovery values for sugars. Supelco-SP2330 wide bore or Supelco-2380 wide bore capillary columns were used. The original work should be examined for details, and manufacturers consulted for best current columns and appropriate operating conditions	35

(*continued*)

Table 5 Continued

Method	Reference.
High-performance liquid chromatography (HPLC) (neutral sugars and uronic acids)	35
The HPLC method described by Englyst et al. (35) used a Dionex system in which internal standard is added to the hydrolyzate, and a guard column (Dionex AG5) is used to prevent sulfate ions from contaminating the analytical column (Dionex Carbopac PA-1, 10 μm, or Dionex Carbopac PA-100, 10 μm, if hexosamines are present). Monosaccharides were detected with pulsed amperometric detection. Gradient elution was performed using a solution of 1.6 mL/L of a 50% (m/v) NaOH solution as the eluent base, with the following elution profile: 0–3.5 min, 23%; 3.5–4.5 min, 23–1% gradient; and 4.5–30 min, 1% (v/v) of the eluent base. Again, the original work (35) should be consulted and manufacturers contacted for best columns currently available and appropriate operating conditions	

It is beyond the scope of this chapter to deal exhaustively with HPLC methods in sugar analysis, especially because a variety of suitable columns, operating conditions, elution profiles, and detection techniques are available that have recently been reviewed (75). However, partly for the purpose of comparison, the GLC and HPLC methods of Englyst et al. (35) are presented together in Table 5.

GLC or HPLC provide information on the monosaccharide composition of the fiber, but require chromatographic equipment. Derivatization for GLC analysis of sugars is now rapid (35,76), but choice of GLC column is important to avoid rapid column deterioration, which can be expensive and lower reliability (77). However, now that HPLC has been successfully used to separate the monosaccharides found in fiber hyrolyzates (35) it is likely to be the preferred method because, although the columns are more expensive, they have a longer life. For routine use in laboratories without GLC or HPLC equipment, colorimetry is satisfactory for measuring dietary fiber (33,34) as long as precautions are taken so that interferences, hydrolysis losses, response differences, and so on, are accounted for (5). Correction factors are necessary to cover losses during hydrolysis, differing derivatization or color yields, and differing responses for the individual sugars in GLC (35,37) and colorimetric (33,34,35,59) analysis of monosaccharides obtained from acid hydrolysis of fiber polysaccharides.

The problem of nonspecificity of reaction in colorimetric methods has required conditions to maximize the desired reaction relative to others, with dichromatic readings for some methods and rigorous exclusion of any extraneous organic matter. Reaction conditions must therefore be strictly controlled. Such methods may be subject to error until practice and skill have been acquired. Therefore, it is advisable to include blanks, standards, and reference samples with each set of unknowns (59).

When measuring fiber polysaccharide colorimetrically from the mixture of monosaccharides in an hydrolyzate, differing color yields of the various monosaccharides may need to be accounted for. When the monosaccharide composition of fiber preparations is not known in advance, but the type of food is, the problem can be largely overcome by using a mixed sugar standard representing the type of sample being analyzed. Englyst and Hudson (34) used a mixture of arabinose–xylose–glucose (3:4:3) for cereals, and arabinose–glucose–galacturonic acid (1:2:1) for fruit and vegetables. If the monosaccharide composition of fiber from a particular food is known, a glucose standard may be used with an appropriate correction factor for the food.

All free sugars in the sample, and most of the protein, should have been extracted from the fiber before it is hydrolyzed for analysis because they can interfere with colorimetric methods. Where sample pre-extraction and digestion produce clean residues that are predominantly cell wall polysaccharide, and the corrections discussed above are able to be applied, colorimetry can give a quantitative and accurate figure for fiber polysaccharide, judging from its agreement with parallel GC determinations (34).

6. Hydrolyzing Fiber Polysaccharides

Acid hydrolysis of fiber polysaccharides to free monosaccharides is usually achieved in a two-step hydrolysis, unless a 1 N H_2SO_4 treatment is used to extract hemicellulose separately from cellulose, as in the earlier fiber methods of Southgate (22) and Englyst

and coworkers (23,68). In the first step, polysaccharides are hydrolyzed with 72% (w/w) (12 M) H_2SO_4 producing sulfated monosaccharides, and, in the second step, the acid is diluted and heated to hydrolyze sulfated to nonsulfated monosaccharides. Some destruction occurs, particularly in the second step (78).

Conditions for optimizing the balance of hydrolysis and destruction over the whole acid treatment have been systematically examined (76,78,79) with the aim of rapid hydrolysis, minimal destruction, and small correction factors. As a result, Englyst recommends the use of 12 M H_2SO_4, 1 h, 35°C, followed by dilution to 2 M H_2SO_4, 100C, 1 h, and has derived the factors that it is necessary to apply to individual monosaccharides before their quantities are added to give an NSP value (78). Hydrolysis conditions of the Uppsala method are slightly different (76), 12 M H_2SO_4, 1 h, 30°C, followed by dilution to 0.4 M H_2SO_4, 125°C, 1 h.

IV. SUMMARY OF MAJOR METHODS FOR DIETARY FIBER ANALYSIS

The methods detailed in this chapter are summarized in Table 3. They may be grouped by the means used for digesting the sample to dissociate nonfiber components from fiber (chemical hydrolysis, detergent/chemical hydrolysis, etc.) and by the ways that the fiber is subsequently measured (gravimetric, colorimetric/gravimetric, etc.).

Space does not permit the methods to be presented fully. The aim is to show the main procedures involved in any method adopted. The descriptions are based on details available in the original publications, which the reader is urged to consult for information on equipment, manipulations, precautions, and various other practical suggestions before applying any of the methods.

A. Points of Procedure

A number of common sense directions that continually recur throughout instructions to the methods for fiber preparation are:

- Include indicative controls to establish enzyme purity and specificity.
- Always run blanks through the analysis to correct for any contribution of reagents and enzymes to measured fiber.
- Weigh appropriate residues before subsampling to enable fiber in the original food to be calculated.
- Precisely standardize sample treatments.
- Mix thoroughly after each addition of reagent.
- Constantly agitate during digestion.
- Avoid lumps during digestion and washing.
- Avoid stranding material on the sides of beakers and tubes.
- Cap tubes.
- Thoroughly suspend residues when washing
- Use porosity 2 grade where sintered glass filters are specified.
- Cool heat-dried residues in a vacuum desiccator or use the hot weighing procedure (29) to avoid moisture contamination.
- Long and variable filtration times can lead to error so use a sample mass that allows the method to proceed smoothly.
- Observe usual safety precautions when using solvents and corrosive chemicals.

B. Methods

1. Faulks and Timms (33)

a. Origin. Derived from the early Englyst GLC procedure (23) but uses DMSO to gelatinize starch and colorimetry to measure sugars.

b. Objective. To provide a robust, reproducible, and rapid method for measuring the NSP component of dietary fiber.

c. Sample Preparation. Freeze dry. Grind to < 1 mm. Extract with boiling 80% EtOH then boiling acetone, each 3 × 25 mL/5 g sample.

d. Enzymes. α-Amylase termamyl 120 L (Novo), diluted 25 × with water. Amyloglucosidase: ex *Aspergillus niger* (Boehringer 102857); use undiluted.

e. Digestion Procedure. To 200 mg sample add 10 mL 0.1 M Tris–maleate buffer (pH 6.7), 100°C, 10 min. Add 0.2 mL of the diluted heat-stable amylase. Digest 100°C, 15 min. Add 40 mL EtOH, 0°C, 30 min. Centrifuge. To the residue add 2 mL DMSO, 100°C, 5 min, then 8 mL 0.1 M acetate buffer (pH 4.6) and 0.1 mL amyloglucosidase. Digest 37°C, 35 min. Add 40 mL EtOH, 0°C, 30 min, centrifuge. Dry the residue. Acid hydrolysis of residue: add 2 mL 12 M H_2SO_4, 35°C, 1 h. Add 22 mL water, heat 100°C, 2 h, removing an aliquot after 1 h for uronic acid determination.

Dilute to volume for colorimetry. Hydrolyzate contains total NSP.

f. Fiber Measurement. Calculate total dietary fiber (TDF) from sugars measured colorimetrically in hydrolyzate; neutralize reducing sugars by *p*-hydroxybenzoic acid hydrazide reaction (71), uronic acids by dimethylphenol reaction (72) (Table 5).

g. Fiber Components Measured. Soluble and insoluble NSPs.

h. Comments. The method is specific for NSPs—lignin, resistant starch, and oligosaccharides are not measured. The method is similar to Englyst's 1982 method (28), but is more rapid through the use of DMSO/heat-stable amylase to digest starch. In foods such as fruit and vegetables, that contain large amounts of pectin, a separate analysis of uronide is required. Results correlate well with GLC measurements but are slightly higher.

2. Englyst and Hudson (34)

a. Origin. Developed from an earlier Englyst procedure (23) for measuring NSP by GLC after acid hydrolysis.

b. Objective. To measure NSP by a simple, rapid colorimetric procedure suitable for routine analysis and automation, where details of monosaccharide composition are not required.

c. Sample Preparation. As is or freeze-dry. Mill if $<10\%$ water, homogenize or freeze-dry and mill if $>10\%$ water. Defat with acetone 40 mL/300 mg dry matter if $>5\%$ fat and/or >10–15% water. Reduce sample particle size to 0.5 mm or less by milling if dry, or by homogenizing then freeze-drying if wet.

d. Enzymes. α-Amylase pancreatin; two α-amylase capsules (9000 BP units, 350 mg pancreatin powder/capsule) and 9 mL water, mix 10 min, centrifuge 10 min, 1500 *g*, use supernatant. Pullulanase: Boehringer 108944 diluted 1:100 in water just before use.

e. Digestion Procedure. To two 100–200 mg dry samples add 2 mL DMSO, heat 100°C, 1 h. Add 8 mL 0.1 M acetate buffer pH 5.2, 50°C. Add 0.5 mL α-amylase solution and 0.1 mL pullulanase. Digest at 42°C, 16 h.

TNSP: to first sample add 40 mL EtOH, stand 1 h, room temperature, centrifuge, wash residues $2 \times 85\%$ EtOH, 40 mL acetone and dry at 65–70°C (R1).

INSP: to second sample add 40 mL 0.2 M phosphate buffer (pH 7.0), heat 100°C, 1 h with stirring. Centrifuge, wash residue with 50 mL phosphate buffer, 50 mL 85% EtOH, 40 mL acetone. Dry residue at 65–70°C (R2).

R1 and R2 hydrolysis: Add 2 mL 12 M H_2SO_4, 35°C, 1 h, then add 22 mL water, 100°C, 2 h.

f. Fiber Measurement. Based on measurement of reducing sugars released from NSP by acid hydrolysis. Total NSP and insoluble NSP calculated from colorimetric measurement of sugars in hydrolyzates, neutral sugars by the dinitrosalicylate–*p*-hydroxybenzoic acid hydrazide (PAHBAH) reaction, and uronic acids measured by the dimethylphenol reaction (Table 5). Soluble NSP = total NSP − insoluble NSP.

g. Fiber Components Measured. NSPs. Total and insoluble NSPs are measured in separate samples. Soluble NSP = total NSP − insoluble NSP.

h. Comments. Suitable for rapid routine analysis. Gives results close to those obtained by GLC. Can be used to measure total nondigestible polysaccharide (NSP plus resistant starch) by omitting DMSO treatment from the dispersion of starch. However, because the sample is ground the resistant starch may be only a portion of that which resists digestion in the gut in foods as normally consumed.

3. Englyst et al. (35)

a. Origin. Developed from the original Southgate method through a series of modifications to improve the specificity and accuracy of determination.

b. Objective. To measure NSPs as a specific food component, and as an index of dietary fiber.

c. Sample Preparation. Reduce sample particle size to 0.5 mm or less by milling if dry or homogenizing then freeze-drying if wet.

d. Enzymes. "Enzyme solution 1": α-amylase: Termamyl (Novo). Use 2.5 mL in 200 mL 0.1 M Na acetate buffer (pH 5.2).

"Enzyme solution 2": 1.2 g pancreatin α-Amylase/protease; (Paynes and Byrne) and 12 mL water, mix 10 min, centrifuge 10 min, 1500 *g*, and take 10 mL supernatant and add 2.5 mL pullulanase (Boehringer 108944).

e. Digestion Procedure. To two samples of maximum 300 mg dry matter add 2 mL DMSO, 100°C, 30 min. Add 8 mL "enzyme solution 1." Digest 100°C,

10 min, cool to 50°C, add 0.5 mL "enzyme solution 2." Digest 50°C, 30 min then 100°C, 10 min.

TNSP: To first sample add 40 mL EtOH, 0°C, 30 min. Centrifuge, wash residue with 50 mL 85% EtOH, 50 mL EtOH, 20 mL acetone. Dry at 80°C (R1).

INSP: Add 40 mL phosphate buffer (pH 7.0), heat 100°C 30 min, room temp 10 min. Centrifuge, wash residue with 50 mL water, 50 mL EtOH, 30 mL acetone. Dry completely at 80°C (R2).

R1 and R2 hydrolysis: Add 5 mL 12 M H_2SO_4, leave at 35°C 1 h, then add 25 mL water and heat 100 C, 1 h.

f. Fiber Measurement. Total NSP and insoluble NSP calculated from sugars measured in hydrolyzates of R1 and R2; neutral sugars by GLC of their alditol acetate derivatives or by HPLC (Table 5), and uronic acids colorimetrically by the dimethylphenol reaction (Table 5). Neutral sugars may also be measured colorimetrically as reducing sugars by the dinitrosalicylate reaction (Table 5).

g. Fiber Components Measured. NSPs. Total and insoluble NSPs are measured in separate samples. Soluble NSP = total NSP − insoluble NSP.

h. Comments. Extraction of soluble NSP involves hot phosphate buffer, pH 7, and extracts more soluble NSPs, particularly pectic substances, than would be extracted under gut conditions from pectin-rich foods. Corrections are made for monosaccharide losses during hydrolysis but are estimates.

Resistant starch is removed by the DMSO treatment and subsequent digestion, and oligosaccharides by the 80% ETOH. Adding resistant starch, oligosaccharides and lignin determined separately to TNSP measured on the same sample will fully account for dietary fiber as most recently defined (3).

4. Monro (36)

a. Origin. A modification of the Englyst colorimetric procedure in which smaller volumes and physiological conditions for soluble fiber extraction are used. A single tube procedure in which the physiological digestion in 12 mL is followed by analysis of undissolved NSP in the same tube, using the Englyst method (method 3, above) scaled down fourfold.

b. Objective. To obtain a measurement of NSP potentially soluble or insoluble under physiological conditions. To measure physiologically soluble fiber in foods eaten raw, and to measure the effects of food processing on fiber solubility, as all other methods cook the foods during starch gelatinization at an early stage in analysis and, therefore, obliterate effects of rawness and processing.

c. Sample Preparation. As is or freeze-dry. Mill if < 10% water, homogenize or freeze dry and mill if > 10% water. Acetone 40 mL/300 mg dry matter if > 5% fat and/or > 10–15% water.

d. Enzymes. α-Amylase/protease pancreatin (Sigma P7000); dissolve 0.5% w/v in intestinal buffer, centrifuge, use supernatant. Protease: pepsin (Sigma P7000). Pepsin and pancreatin are used during physiological extraction of soluble NSP, subsequent steps use enzymes specified in Englyst procedure.

e. Digestion Procedure. The digestion is carried out in screw-cap glass tubes capable of holding 12 mL and being centrifuged. Wash 100–200 mg (dry) sample with 12 mL gastric buffer (0.05 M HCl/54 mM NaCl), pH 2.0, 37°C, 10 min. Centrifuge.

Remove supernatant. To the residue add 12 mL gastric buffer and 20 mg pepsin. Digest 37°C, 2 h. Centrifuge, wash the residue with 12 mL intestinal buffer (per L: 8 g NaCl, 0.2 g KCl, 0.26 g $MgSO_4 \cdot 7H_2O$, 0.06 g $NaH_2PO_4 \cdot 2H_2O$, 1 g $NaHCO_3$, 0.2 g $CaCl_2$), centrifuge. To the residue add 12 mL 0.5% (w/v) pancreatin in intestinal buffer (centrifuged supernatant). Digest 2 h, 37°C. Centrifuge, wash with 2 × 12 mL intestinal buffer, freeze dry. Analyse residue and an undigested sample for total NSP by the Englyst procedure.

f. Fiber Measurement. Physiological soluble NSP = TNSP in undigested sample − TNSP in digested sample.

g. Fiber Components Measured. Physiologically soluble and physiologically insoluble NSP.

h. Comments. Introduces physiological relevance by extracting and separating SNSP under physiological conditions before digesting starch. Circumvents effects of heat and DMSO during digestion on polysaccharide distribution between SNSP and INSP. Gives a measure of dietary fiber potentially solubulized under gut conditions, from foods eaten raw, so can be used to measure effects of food processing/cooking on NSP solubility under gut conditions, and to give a relevant SNSP value for raw foods. As milled samples are used, it measures fiber potentially soluble in the gut rather than that which would be extracted from foods as normally swallowed.

5. Uppsala (AOAC Method 994.13) (37,76)

A detailed description of the Uppsala method with a critical discussion of various steps and alternative methods, and a comparison with the NSP method of Englyst has been published (76). The Uppsala method, like that of Englyst, has been modified very little in the last decade.

a. Origin. Arose from a background of carbohydrate chemistry, applied to the analysis of polysaccharide constituents of dietary fiber.

b. Objective. To improve accuracy of dietary fiber measurement as nondigestible polysaccharides and lignin by measuring nondigestible polysaccharides directly as their constituent monosaccharides, avoiding some of the inaccuracies in gravimetric methods arising from the presence of noncarbohydrate, nonlignin materials in fiber residues.

c. Sample Preparation. As is or dry. Mill if dry, homogenize if wet. If >6% fat, sonicate 1–3 g in 80% EtOH, 15 min, 3 × 75 mL, centrifuge, and extract with petroleum ether or hexane 10 min, 2 × 50 mL.

d. Enzymes. α-Amylase heat stable, Termamyl 120 L (Novo Nordisk); use undiluted. Amyloglucosidase: Boehringer Mannheim (Cat. No. 1202367; 140 EU/mL), ex *Aspergillus niger*; use undiluted.

e. Digestion Procedure. Suspend 1.5–2.0 g dry pre-extracted sample (1 g if starch rich) in 75 mL Na acetate buffer (pH 5.0), add 0.1 mL heat stable amylase. Digest 100°C, 30 min. Cool to 60°C, add 0.5 mL amyloglucosidase. Digest 60°C, 16 h.

TDF: add 4 vol EtOH, 4°C, 0.5 h. Centrifuge, wash residue 2 × 80 mL 80% EtOH, 50 mL acetone, dry in warm air.

Where Separation of TDF into SDF, IDF Required. Centrifuge digestate, retain supernatant (S) wash residue (R) with 2 × 50 mL water.

SDF: Combine supernatant (S) with water washings, concentrate to 100 mL, dialyse versus tap water (1 × 24 h), distilled water (1 × 24 h), freeze-dry and weigh.

IDF: wash residue (R) with 2 × 50 mL EtOH, 50 mL acetone, dry in warm air.

f. Fiber Measurement. Hydrolyses: Grind a subsample (150–250 mg) of each fiber preparation, hydrolyze with 3.0 mL 12 M H_2SO_4, 30°C, 1 h. Add 79 mL water, 5 mL myoinositol (3 mg/mL, internal standard), and autoclave 125°C, 1 h. Klason lignin = dry weight of residue from 12 M H_2SO_4 hydrolysis of IDF.

Measure neutral sugars in hydrolyzed fractions (SDF and IDF, or TDF) by GC of their alditol–acetate derivatives. Measure uronic acids in a separate subsample of fiber preparation by potentiometry, in which potentiometric changes are measured when CO_2 released by refluxing in hydroiodic acid under nitrogen is passed through a $KI/CdSO_4$ solution and collected in a cell containing 0.02 M NaOH. Alternatively, the colorimetric dimethylphenol (DMP) reaction for uronic acids may be used (Table 5) (72). Calculate neutral sugar and uronic acids as polysaccharides. Klason lignin = dry weight of residue from 12 M H_2SO_4 hydrolysis. IDF and SDF = neutral sugar + uronic acid + Klason lignin in fraction; TDF = neutral sugar + uronic acid + Klason lignin.

g. Fiber Components Measured. Soluble and insoluble NSPs, some resistant starch, plus Klason lignin.

h. Comments. Well-researched single-tube GLC method. Potentiometric analysis of uronic acids is specific but slow and requires specialized equipment, but colorimetric analysis can be used alternatively. Results are similar to, but often lower than, those obtained with the Prosky (AOAC) gravimetric method for TDF. The soluble fiber is nonphysiological, as is the resistant starch component in the TDF and IDF.

6. Mongeau and Brassard (AOAC Method 992.16, AACC Method 32-20) (29)

a. Origin. Derived from neutral detergent methods (4) by including amylase digestion and separate measurement of a soluble dietary fiber fraction to overcome the presence of residual starch in the detergent residue, and loss of soluble fiber, in detergent fiber methods.

b. Objective. To obtain a clean, low-protein fiber residue by neutral detergent extraction of solubles. Total dietary fiber is obtained by adding separately measured insoluble (neutral detergent) fiber (NDF) and soluble fiber (SDF) fractions.

c. Sample Preparation. Freeze-dry. Grind to pass a 20 mesh (0.075 mm^2) screen. Acetone, room temperature 1 h, if >5% fat.

d. Enzymes. α-Amylase Sigma A-6880; use 5% in 0.1 M phosphate buffer, pH 7.0. Amyloglucosidase: Sigma A-9268; use 15% v/v in 2.0 M Na acetate–acetic acid buffer, pH 4.5.

e. Digestion Procedure. NDF: Reflux 0.5–1 g sample in neutral detergent (per L: 18.61 g Na_2EDTA, 6.81 g Na borate decahydrate, 30 g Na lauryl sulfate, 10 mL 2-ethoxyethanol, 4.56 g separately dissolved Na_2HPO_4; with 2 mL decahydronaphthalene added just before boiling) for 1 h, filter in tared crucible, add 10 mL α-amylase solution and 15 mL hot water, stand 5 min, filter, plug crucible and add further 10 mL amylase and 15 mL hot water, digest 55°C, 60 min. Filter, wash 3 × water, 2 × acetone. Dry 100°C overnight.

SDF: heat 0.5 g sample in 20 mL water 100°C, 15 min. Cool to 55°C, and 2 mL amyloglucosidase, digest 55°C, 1.5 h, reheat 100°C, 30 min, filter, wash with 10 mL hot water. Add 2 mL amyloglucosidase to filtrate plus washings. Digest 55°C, 1 h. Add 4 vol. EtOH, stand 1 h, filter in tared 10–15 μm pore Gooch crucible, wash 2 × 75% EtOH, 2 × acetone. Dry 100°C overnight.

f. Fiber Measurement. IDF (= NDF): weigh crucible plus residue. Ash at 525°C, 4 h. Hold at 100°C overnight, weigh hot. IDF = loss in weight on ashing.

SDF: weigh crucible plus residue, and blank. Ash and weigh as for IDF (above). SDF = loss in weight − loss in weight of blank.

g. Fiber Components Measured. Lignin and most cell wall polysaccharides as a complex, but some soluble fibers will not be recovered. Oligosaccharides and resistant starch are not measured.

h. Comments. The hot neutral detergent treatment used to prepare neutral detergent fiber is likely to extract more soluble fiber, and certainly more pectin, than the hot water used to extract the soluble fiber fraction. Therefore, NDF plus SDF may not account for all of the NSPs present. The method is rapid and shows good precision. Most suitable for cereal foods low in pectin.

7. Asp et al. (38)

a. Origin. Derived from earlier enzymatic–gravimetric methods of Furda et al. (80), Schweizer and Würsch (81), and Asp and Johansson (82), which used long digestion times and laborious centrifugations to recover fiber.

b. Objective. To provide a simple and rapid method for separating soluble and insoluble fiber from protein and starch by digesting the protein and starch with specific enzymes, precipitating the soluble fiber with ethanol, and collecting it by rapid filtration on celite.

c. Sample Preparation. Homogenize and freeze-dry. Mill to 0.3 mm. Extract with petroleum ether 40 mL/g sample, room temperature, 15 min.

d. Enzymes. α-Amylase (heat stable): Termamyl 60 L (Novo); use undiluted. Amylase/protease: pancreatin (Sigma, 4 × NF); used as is. Protease: pepsin NF (Merck); used as is.

e. Digestion Procedure. Suspend 1 g sample in 25 mL 0.1 M phosphate buffer, pH 6.0. Add 0.1 mL heat-stable amylase, digest 100°C, 15 min. Cool, add HCl to pH 1.5 plus 100 mg pepsin, digest 40°C, 60 min. Add 20 mL water, NaOH to pH 6.8, and 10 mg pancreatin. Digest 40°C, 60 min. Add HCl to pH 4.5.

TDF: add 4 vol. 95% EtOH, 60°C, 1 h. Filter through a weighed crucible containing 0.5 g Celite. Dry 105°C overnight.

SDF, IDF: filter digest as for total fiber but without prior addition of EtOH, wash residue with 2 × 10 mL water.

SDF: to filtrate plus washings add 4 vol. EtOH, 60°C, stand 1 h. Collect precipitate on Celite, wash with 2 × 10 mL each of 78% EtOH, 95% EtOH, acetone. Dry 105°C overnight.

IDF: wash residue with 2 × 10 mL each of 95% EtOH and acetone. Dry 105°C overnight.

f. Fiber Measurement. SDF and IDF: weigh dry residue plus crucible, ash at 550°C, 5 h. Fiber = weight (crucible + residue) − weight (crucible + ash) − weight ash-free reagent blank − protein determined in a separate sample.

g. Fiber Components Measured. Most soluble and insoluble NSPs plus lignin. Oligosaccharides are not measured and only some of the resistant starch.

h. Comments. This method is very similar to, and a forerunner of, the Prosky method for TDF (AOAC Method 985.29) (41) and IDF (AOAC Method 991.42) plus SDF (AOAC Method 993.19) methods (26). The soluble fiber fraction is unlikely to represent fiber soluble in the gut because the extraction conditions are unlike those in the gut.

8. Urea Enzymatic Dialysis I (39)

a. Origin. Based on the AOAC TDF method, but radically modifies it to achieve a low starch, low protein, low ash dietary fiber residue, without the need for heat, by using 8 M urea as the hydrating medium.

Simplifies TDF analysis as it uses only one solution and two enzymes.

b. Objective. To provide a simple and economical measure of TDF without the need for large corrections for protein and ash required by the AOAC TDF method.

c. Sample Preparation. Freeze-dry. Dry mill to 0.5–1 mm. Extract with petroleum ether, 3×25 mL/10 g sample if $>10\%$ fat.

d. Enzymes. α-Amylase (heat stable): Sigma A-0164; use 0.67% (v/v) in 8 M urea. Or use Novo 240 L, 0.33% v/v in 8 M urea. Protease: Savinase (Novo 8.0 L); used as is.

e. Digestion Procedure. Weigh 0.9–1 g sample into dialysis tubes pretreated with 10% EtOH, add 30 mL 0.67% heat-stable amylase in 8 M urea, digest at room temperature 3.5–4.5 h. Add 0.5 mL protease (Savinase), digest 50°C, 2–28 h, with continuous water exchange. Empty into a container, add 4 vol. EtOH, stand 4 h. Filter through tared Whatman 54 filter paper. Wash with 80% EtOH. Dry 105°C, 8 h.

f. Fiber Measurement. Weigh filter papers plus residues, and filter paper blanks. Analyze a set of duplicates for protein by the Kjeldahl method, correcting for N in a filter paper blank. Ash other duplicates and blanks at 525°C for 8 h.

TDF = (weight paper + residue) − (weight protein in residue + weight ash in residue) − weight blank paper.

g. Fiber Components Measured. Lignin plus NSPs with a possibility of other insoluble unidentified matter.

h. Comments. TDF method. Urea hydrates and extracts starch and soluble polysaccharides without heat damage. Lower ash, protein, and resistant starch in the residue than in Prosky AOAC TDF method. Rapid and inexpensive.

9. Urea Enzymatic Dialysis II (40)

a. Origin. Modification of Urea Enzymatic Dialysis I (UEDI), (method 8, above).

b. Objective. To obtain separate measurements of SDF and IDF with low starch, and small corrections for protein and ash.

c. Sample Preparation. As for UEDI.

d. Enzymes. As for UEDI but Termamyl 120 L (Novo) used to prepare 0.33% (v/v) heat-stable amylase–8 M urea solution.

e. Digestion Procedure. As for UEDI (above) until 2–28 h dialysis completed, then filter through tared Whatman 54 filter paper, wash with water.

SDF: to filtrate plus washing add 4 vol. EtOH, stand 4 h, filter through tared filter paper. Dry precipitate on paper 105°C, 8 h.

IDF: wash residue from dialysis with 80% EtOH. Dry 105°C, 8 h.

f. Fiber Measurement. As for UEDI (above), but with separate sets of papers for SDF and IDF, treated in the same way, to obtain protein and ash corrections for each.

Fiber = weight (residue + paper) − (weight protein in residue + weight ash in residue) − weight blank paper.

g. Fiber Components Measured. Soluble fiber and insoluble fiber consisting of lignin plus NSPs with unidentified matter possible. Oligosaccharides and resistant starch not measured.

h. Comments. Modified UEDI (above), used to separate soluble and insoluble dietary fiber. The low-temperature conditions for starch digestion are likely to extract some polysaccharides to a lesser degree than in methods (such as AOAC and Englyst) that use hot buffer. The conditions of extraction differ from gut conditions so the physiological relevance of the urea enzymatic soluble fiber is as questionable as that of the Prosky and Englyst methods.

10. Prosky TDF (AOAC Method 985.29) (41)

a. Origin. Developed from several similar lines of development of fiber analysis [Asp et al. (38), Furda (80), and Schweizer and Würsch (81)] brought together, incorporating thermostable amylases, ethanol precipitation of soluble fiber, and Celite filtration of the fiber residues.

b. Objective. Rapid and practical enzymatic–gravimetric determination of total dietary fiber for the purposes of food regulation.

c. Sample Preparation. Vacuum oven, 70°C, overnight. Dry mill to pass 0.3–0.5 mm. Extract with petroleum ether 3×25 mL/g sample if $>5\%$ fat.

d. Enzymes. Heat stable α-amylase: Termamyl 120 L (Novo); use undiluted. Amyloglucosidase: Sigma A 9268; use undiluted. Protease: Subtilopeptidase A (Sigma type VIII); use as is.

The foregoing enzymes were used in the original method but their equivalents are available in various TDF kits and enzymes specifically for TDF measurement, such as from Megazyme International Ireland Ltd. (Bray, Eire) and Sigma (St. Louis, MO).

e. Digestion Procedure. To duplicate 1 g samples add 50 mL 0.05 M phosphate buffer (pH 6) plus 0.1 mL heat-stable α-amylase. Digest 95–100°C, 15 min. Cool, add 0.275 N NaOH to pH 7.5 (about 10 mL) plus 5 mg protease. Digest 60°C, 30 min. Cool, add 0.325 M HCl (about 10 mL) to pH 4.0–4.6, add 0.3 mL amyloglucosidase. Digest 60°C, 30 min. Add 280 mL 95% EtOH at 60°C, stand 1 h. Filter on a tared crucible plus Celite. Wash with 3×20 mL 78% EtOH, 2×10 ml each of 95% EtOH, and acetone. Dry in 70°C vacuum or 105°C air oven. Cool in desiccator and weigh.

f. Fiber Measurement. TDF (in duplicate samples and reagent blanks): weigh crucibles after cooling in desiccator. Determine protein (Kjeldahl N × 6.25) on one set of duplicates. Incinerate second set at 525°C, 5 h. Apparent fiber = weight residue − weight protein − weight ash. Actual TDF = apparent fiber − fiber in blank.

g. Fiber Components Measured. Materials insoluble in 78% ethanol after milling, lipid extraction, and amylase digestion, with allowance made for ash and total protein. Predominantly NSP, lignin, and a portion of resistant starch, but a range of associated materials may add to the fiber weight.

h. Comments. Widely accepted for food labeling. Requires ash and protein correction (with associated error) on residue. Unidentified matter and coprecipitated phosphate in the residue may inflate fiber values (48,64,65). Filtration may be difficult with viscous digests. Procedure modified slightly to give AOAC Method 991.42 (insoluble DF) and 993.19 (soluble DF method), and later, the AOAC method of Lee et al. (AOAC Method 991.43) (27) for total, soluble, and insoluble dietary fiber using MES–Tris buffer to overcome contamination by phosphate. Milling of samples for analysis means that the resistant starch in the TDF is unlikely to reflect levels in the foods as consumed.

11. Prosky SDF (AOAC Method 993.19) Plus IDF (AOAC Method 991.42) (26)

a. Origin. Developed as a modification of the Prosky TDF method (AOAC 985.29) to give separate measurement of insoluble and soluble dietary fiber.

b. Objective. Rapid and practical enzymatic–gravimetric determination of soluble and insoluble dietary fiber for the purposes of food labeling.

c. Sample Preparation. As for previous method (Prosky TDF).

d. Enzymes. As for previous method (Prosky TDF).

e. Digestion Procedure. Duplicate 1 g samples in 50 mL 0.8 M phosphate buffer, final pH 6.0, plus 0.1 mL heat-stable α-amylase, 95–100°C, 15 min. Cool to room temperature, add 10 mL 0.275 M NaOH to pH 7.5. Add 5 mg protease. Digest 60°C, 30 min. Cool, add 10 mL 0.325 M HCl to pH 4.0–4.6, add 0.3 mL amyloglucosidase. Digest 60°C, 30 min.

IDF: Filter on tared crucible plus Celite, wash residue with 2×10 mL each of water, 95% EtOH, acetone. Dry as for SDF.

SDF: adjust supernatant and water washings to 100 g, add 400 mL 95% EtOH at 60°C, stand at room temperature 1 h. Collect precipitate in tared crucible plus Celite, wash with 2×20 mL 78% EtOH, 2×10 mL 95% EtOH, 2×10 mL acetone. Dry in a 70°C vacuum oven or 105°C air oven overnight.

TDF: adjust volume of digest to 100 mL, continue as for soluble fiber, i.e., leave out filtration to separate IDF and SDF.

f. Fiber Measurement. TDF, IDF, SDF (in duplicate samples and reagent blanks): weigh crucibles after cooling in desiccator. Determine protein (Kjeldahl N × 6.25) on one set of duplicates. Incinerate second set at 525°C, 5 h. Apparent fiber = weight residue − weight protein − weight ash.

Actual TDF, IDF, or SDF = apparent fiber − fiber in blank.

g. Fiber Components Measured. As for previous method (Prosky TDF).

h. Comments. The soluble fiber is extracted under nonphysiological conditions so its relevance to nutrition is questionable. As extraction conditions for IDF and SDF are the same, the errors or lack of relevance of SDF are transferred through to the IDF fraction. The TDF or IDF contains a portion of resistant starch (mainly retrograded) that is unlikely to reflect the resistant starch load in foods as consumed.

12. Lee/Prosky TDF, SDF, IDF (AOAC Method 991.43, AACC Method 32-07) (27)

a. Origin. A direct modification of the Prosky method in which phosphate buffer is replaced by MES–Tris buffer to eliminate coprecipitation of phosphate and fiber by ethanol, which inflates fiber values, and remove one pH adjustment.

b. Objective. Rapid and practical enzymatic–gravimetric determination of total fiber or soluble and insoluble dietary fiber, for the purposes of food labeling.

c. Sample Preparation. As for Prosky TDF method (method 10).

d. Enzymes. α-Amylase: heat stable, (a) Sigma A-3306 or Termamyl 300 L (Novo); use undiluted. Amyloglucosidase: (b) Sigma A 9913; use undiluted. Protease: (c) Sigma P3910, use 50 mg/mL 0.05 M MES–Tris buffer, pH 8.2; (a), (b), and (c) are sold in pretested kits (e.g., Megazyme, KTDFR; Sigma TDF-C10).

e. Digestion Procedure. (The original procedure given, but adjustments according to instructions may be required if using a kit.) Disperse duplicate 1 g samples in 40 mL 0.05 M MES/0.05 M Tris buffer, pH 8.2. Add 50 μL heat-stable α-amylase solution, heat 95–100°C, 15 min. Cool to 60°C, add 100 μL protease solution, digest 60°C, 30 min. Add 5 mL 0.561 N HCl, adjust to pH 4.0–4.7 with 1 N NaOH or 1 N HCl at 60°C. Add 300 μL amyloglucosidase, digest 60°C, 30 min.

TDF: add 225 mL 95% EtOH at 60°C, stand 1 h room temperature. Filter through a tared crucible plus Celite, wash with 2 × 15 mL each, 78% EtOH, 95% EtOH, and acetone. Dry in 70°C vacuum oven or 105°C air oven overnight.

SDF, IDF: Filter digestate in tared crucible plus Celite. Wash with 2 × 10 mL water at 70°C.

SDF: to the precipitate plus water washings, add 4 vol. 95% EtOH at 60°C, stand 1 h room temperature. Collect precipitate on Celite and wash with 78% and 95% EtOH, acetone and dry, as for TDF.

IDF: Wash residue with 78 and 95% EtOH, acetone and dry, as for TDF.

f. Fiber Measurement. As for Prosky IDF plus SDF method (method 11 above).

g. Fiber Components Measured. Materials insoluble in 80% ethanol after milling, lipid extraction, amylase digestion, and allowance for ash and total protein. Predominantly NSP, lignin, and a portion of resistant starch, but some unspecified dietary fiber-associated matter occurs in the residue.

h. Comments. Changing the buffer from phosphate to MES/Tris in this modification of the Prosky method eliminates a pH adjustment, reduces the number of steps, averts phosphate precipitation into fiber, and decreases volumes. Faster than the Prosky method, giving similar results but improved precision. The soluble fiber and resistant starch components do not represent those acting in vivo because extraction conditions are not physiological.

13. Li and Andrews (42)

a. Origin. The Prosky method modified by autoclaving sample, and omitting the protease, thereby eliminating pH adjustment.

b. Objective. More rapid and simpler measurement of dietary fiber than the Prosky and Lee methods.

c. Sample Preparation. Freeze dry. Mill to pass 40 mesh. Extract with hexane if > 5% fat.

d. Enzymes. Amyloglucosidase: Sigma A 9913 or equivalent, such as Megazyme amyloglucosidase (E-AMGDF); use 0.15 mL amyloglucosidase plus 1 mL 4 M Na acetate buffer (pH 4.8) plus 0.85 mL water/2 mL enzyme solution.

e. Digestion Procedure. To duplicate 0.5 g samples add 25 mL deionized water, heat 121°C, 75 min, or autoclave 1 h. Cool to 65–70°C, add 2 mL amyloglucosidase solution. Digest 55°C, 2 h. Make to 100 mL with 95% EtOH, stand 1 h. Filter in a tared crucible plus Celite. Wash with 78 and 95% EtOH, and acetone, 2 × 10 mL of each. Dry 105°C overnight.

f. Fiber Measurement. As for Prosky IDF plus SDF method (method 11 above).

g. Fiber Components Measured. Materials insoluble in 80% ethanol after milling, lipid extraction, amylase digestion, and allowance for ash and total protein. Predominantly NSP, lignin, and a portion of resistant starch, but some nonspecified matter occurs in the residue.

h. Comments. A simple rapid method as it uses autoclaving, one enzyme (no protease), one buffer, no pH adjustments. May heat-degrade fiber. Soluble fiber of doubtful physiological significance. Residue protein

in high-protein, low-fiber food may add to correction error.

14. Li and Cardozo I (5,43)

a. Origin. Modification of the method of Li and Andrews (method 13) by filtering to separate soluble and insoluble fiber.

b. Objective. Rapid measurement of soluble and insoluble dietary fiber in foods containing ≥10% total dietary fiber and ≤ 2% starch.

c. Sample Preparation. As for method 13 above (Li and Andrews).

d. Enzymes. As for method 13 above (Li and Andrews).

e. Digestion Procedure. As for method 13 above (Li and Andrews), but filter after amyloglucosidase digestion, before EtOH addition.

IDF: rinse residue with 35 mL 95% EtOH, acetone. Dry 105°C overnight.

SDF: combine filtrate and 95% EtOH washing, add 100 mL 95% EtOH, stand 1 h, filter, rinse precipitate with EtOH and acetone. Dry 105°C overnight.

f. Fiber Measurement. As for Prosky IDF plus SDF method (method 11 above).

g. Fiber Components Measured. Materials insoluble in 80% ethanol after milling, lipid extraction, amylase digestion, and allowance for ash and total protein—NSP, lignin, plus some unidentified matter.

h. Comments. Method 13 (Li and Andrews) modified to measure SDF and IDF. Autoclaving makes the physiological significance of the SDF doubtful.

15. Li and Cardozo II (AOAC Method 993.21) (5,28)

a. Origin. Modification of method 14 (Li and Cardozo I) so that TDF is measured and no digestive enzymes are used.

b. Objective. Rapid measurement of TDF in foods containing ≥10% total dietary fiber and ≤ 2% starch.

c. Sample Preparation. As for method 14 (Li and Cardozo).

d. Enzymes. Enzymes not used.

e. Digestion Procedure. Extract duplicate 500 mg freeze-dried samples with 25 mL deionized water, stand 37°C, 90 min. Add 100 mL 95% EtOH, stand at room temperature 1 h. Filter in a tared crucible plus Celite, wash 2 × 20 mL 78% EtOH, 2 × 10 mL 95% EtOH, 10 mL acetone. Dry 105°C, 2 h.

f. Fiber Measurement. As for Prosky IDF plus SDF method (method 11).

g. Fiber Components Measured. Materials insoluble in 80% ethanol, with ash and total protein subtracted.

h. Comments. The Prosky method reduced to simple water extraction, without protease or amylase digestion, so is suitable only for materials such as fruit and vegetables, which contain a relatively high proportion of dietary fiber, and little starch and protein.

V. PROBLEMATIC FIBER COMPONENTS

Resistant starch, soluble fiber, lignin, and 80% ethanol-soluble components are all problematic in some way: resistant starch because it has seldom been adequately measured in dietary fiber analysis, soluble fiber because the way it is measured in most methods makes it of doubtful physiological relevance, lignin because it is not usually true lignin that is measured, and 80% ethanol-soluble non-digested carbohydrates because they have not been accounted for in most dietary fiber analyses.

A. Resistant Starch

Resistant starch (Table 1) is a minor component in most highly processed foods in which native food structure has been destroyed and starch gelatinized (53), unless added as a dietary fiber ingredient. However, it may contribute significantly to nondigestible polysaccharides in starchy foods such as whole and kibbled grains, which retain enough structure to inhibit amylase access to starch during digestion, or when limited gelatinisation has occurred. Resistant starch has been classified into four types:

1. RS_1: physically protected or encapsulated, as in whole and partly milled grains.
2. RS_2: raw starchy foods, such as potatoes and green banana.

3. RS_3: retrograded starch that has been gelatinized and then cooled.
4. RS_4: starch in which chemical modification confers amylase resistance.

In dietary fiber analyses, such as those of Prosky et al. (41) and Theander et al. (Uppsala) (37), resistant starch in the form of retrograded starch may be present in the dietary fiber isolated, but it is, however, only a portion of the resistant starch load consumed in most foods. The reason is that usual sample comminution in preparation for analysis, eliminates the normal food structural impediments that lead to RS_1 and this is the most important cause of starch indigestibility in vivo (83). The resistant starch fraction measured by the Prosky and Theander methods is, therefore, of doubtful physiological meaning, even though measurement of resistant starch has been used as an argument in support of such methods (37).

Because resistant starch content can be so variable, as a result of food manufacture, preparation, storage, and eating processes, and because the conditions of resistance to digestion have not been physiological in dietary fiber methods, some food analysts, such as Englyst, consider that resistant starch should be excluded from dietary fiber analysis. Englyst prefers measuring resistant starch as an important food constituent in its own right, with fiber measured separately as NSP so that it can act as a valid index of plant cell walls in a food (49). One of the aims of the DMSO enzymatic–chemical method, for measuring nonstarch polysaccharide, was to overcome the enormous variability in fiber composition and intake imposed by the effects of food processing and individual chewing patterns on resistant starch intake.

However, with the amount of evidence accumulated in recent years to show that resistant starch is an effective prebiotic (84) that occurs in significant amounts in the diet, both naturally and added as an ingredient, the consensus is that it should be measured as part of the dietary fiber complex (3,19). Also, because dietary fiber is considered a physiological as well as a chemical concept, it is considered that the resistant starch load contributed by a food as it would reach the intestine in vivo should be measured. Therefore, the analytical requirements that may be imposed by the latest definition of dietary fiber (3) fit well with the methods of Englyst for measurement of NSP (35), and total resistant starch (58), even if not with the definition of dietary fiber as NSP.

Accurate, standardized measurement of resistant starch is a formidable challenge because it requires that sample preparation and analysis replicate the physical factors that determine food particle breakdown in vivo, including both chewing and peristaltic actions in the gut. Chewing is a complex process to mimick and standardize, and one wonders why such rigid criteria are not applied to the measurement of other nutrients for food labeling because food structure can inhibit digestion or release a wide range of food components in addition to carbohydrates (14). In fact, because so many noncarbohydrate materials enter the colon, with positive effects, it has recently been proposed that dietary fiber should not be regarded as a carbohydrate component, but as "Any dietary component that reaches the colon without being absorbed in a healthy human gut" (14).

Several methods have been used in an attempt to provide a realistic measurement of resistant starch, some using subjects to chew the samples (50,51), others using mechanical processes such as grinding (52–54), and some also include mechanical processes during digestion (53).

Formation of resistant starch during analysis should of course be avoided, as it would lead to overestimation of the nondigestible polysaccharide that was present in the food as consumed, before analysis. Dehydration may encourage resistant starch formation so, ideally, samples should be analyzed for dietary fiber or NSP in the form in which they are eaten. However, because of the practical and statistical benefits of dealing with a dry homogeneous powder (85), fiber analyses mostly use dried, milled, samples.

Although resistant starch is digested after being gelatinized with hot DMSO in methods measuring fiber as NSP (Table 3), concern has been expressed that the distribution of polysaccharides between soluble and insoluble fiber fractions is affected by the DMSO treatment (5). A limited study of the Englyst procedure with macaroni and peas, however, did not detect any effect of DMSO on polysaccharide distribution (63).

B. Soluble Fiber

1. Relevance and Standardization

Interest in measuring soluble and insoluble components of fiber intensified as evidence accumulated that the different physical properties of the two fractions might be linked to health. However, analysis of soluble fiber has been marred by lack of standardization and lack of physiological relevance (36).

Most methods for soluble fiber analysis are simply modifications of total-fiber methods, with a step inserted after starch digestion to separate soluble from insoluble material by filtering or centrifuging the digest. The soluble fiber is then recovered from the liquid phase, most often by precipitating with ethanol. There are three important consequences of adopting such an approach:

1. Soluble fiber values are of doubtful nutritional relevance when the fiber is solubilized under the relatively harsh conditions normally used for starch gelatinisation and digestion, rather than under physiological conditions.
2. Soluble fiber values vary from method to method because conditions for extracting starch/soluble fiber are not standardized. Methods differ in both pH and buffer species, both of which can have a powerful effect on the solubility of pectic substances (49,62), which are often a major component of soluble fiber.
3. True differences in soluble fiber values for a food before and after cooking, and for foods eaten raw will be largely obliterated in methods that apply a heat treatment when gelatinizing/digesting starch, as the food, in effect, becomes "cooked" during analysis.

The heat treatments that samples are almost invariably subjected to, in order to gelatinize starch prior to starch digestion, mean that there are a large number of rather meaningless soluble fiber results in food databases. Worse, however, it means that there are no physiologically relevant data for fruit and vegetables eaten raw, and there are no data that can show the effects of food processing on soluble fiber because such effects are obliterated during analysis.

If soluble fiber is extracted under simulated standard gastrointestinal conditions, and recovered before the residue is subjected to starch digestion, the influence of nonphysiological conditions on the distribution of polysaccharides between the soluble and insoluble fractions is avoided (Method 4, Table 3) (36). A physiological digestion can be put at the front end of any total fiber method to give a "nutritionally valid" measure of soluble fiber, and remaining fiber (i.e., physiologically insoluble) measured with a TDF analysis of the digested residue. The gastrointestinal conditions can be changed to align the method with gut physiology in any group of interest.

If soluble fiber values are to be used to predict fiber soluble in the gut, as has been the case in clinical and nutritional research, it makes sense first to define the relevant internal conditions and then to use them to extract the soluble fiber. The gut milieu is extremely complex, and the solubility of fiber is likely to be affected by other food components associated with it, and by various properties of the food that may be expressed differently under different conditions (86). Therefore, the only way to be sure that soluble fiber measured during analysis in vitro represents that soluble in vivo, is to extract under conditions dictated by the gut rather than by analysts. Then, the idiosyncratic response of a food to analytical conditions will not give rise to problems of nutritional validity.

Recent methods for dietary fiber analysis have moved away from the soluble–insoluble fractionation because it is felt that fiber solubility per se is less important to health than the properties of the dietary fiber in solution. However, if the type of dietary fiber in a product is known, and its properties, there are likely to be occasions in which a knowledge of the amount of fiber solubilized would be useful, as long as physiological conditions are used.

Part of the reason for abandoning soluble fiber analysis is, no doubt, that it has seldom been appropriately measured, so it not surprising that expected associations between levels of soluble fiber in foods and physiological effects have often not been obtained.

C. Lignin

Measurement of lignin has recently been reviewed (55), and will be dealt with here solely in the context of dietary fiber analysis. As a component of plant cell walls, true lignin is usually considered a part of dietary fiber, but it is not included in some dietary fiber methods and is not measured as true lignin in the dietary fiber methods that do include it.

Due to the difficulty of measuring true lignin as a discrete food component, all dietary fiber methods that measure "lignin," measure "Klason lignin," which is the weight of residue remaining after 72% (w/w) (12 M) H_2SO_4 hydrolysis of the insoluble fiber residue, as measured in the Uppsala method (Table 3) (37).

"Acid detergent lignin" (ADL) is another gravimetric measure of lignin that is often used as an alternative to Klason lignin. Ground samples are refluxed, 1 g of sample in 100 mL of 0.5 M H_2SO_4–2% cetyltrimethylammonium bromide, for 60 min, to obtain a low-protein residue consisting largely of lignin and cellulose, known as acid detergent fiber (87). The washed, dried residue, is treated with cool (15°C) 72% H_2SO_4 for 3 h, and again filtered, washed, dried, and

weighed. ADL is the weight of residue minus its ash content (AOAC Method 973.18 D for ADL).

Both Klason lignin and ADL are only approximations to true lignin because, on the one hand, losses of lignin occur in the acid treatment, and on the other, because unhydrolyzed polysaccharide residues, Maillard products, tannins, and cutins contribute to the residue weight (55).

One reason for the preference for dietary fiber to be limited to NSP in the UK was the difficulty of obtaining a measure of true lignin (48,49). Lignin is a small component in most foods and is subject to large errors when measured as apparent lignin in the form of acid hydrolysed residues, which contain many forms of pseudolignin. For instance, heat treatments of foods containing carbohydrates and proteins, both before and during analysis, may inflate the amount of apparent lignin through the formation of Maillard products. If lignin is suspected of being a significant component it can, like resistant starch, be measured separately.

As an alternative to direct measurement of Klason lignin or ADL gravimetrically, an indirect method is available in which lignin is measured by difference, as the loss in weight on permanganate oxidation of acid-detergent fiber (88). The method has been recommended because it measures fewer artifacts as lignin, is shorter, and uses reagents that are less corrosive than 72% H_2SO_4 (59).

A colorimetric procedure based on reaction with acetyl bromide is also available, but has not been used in the analysis of dietary fiber per se (89).

D. Fiber Components not Precipitated by 80% Ethanol

As the definition of dietary fiber has been extended to include nondigestible carbohydrates, notably oligosaccharides, beyond those that are part of plant cell wall structure, 80% ethanol precipitation has become less universally appropriate for the recovery of soluble dietary fiber. In particular, a number of nondigestible, 80% ethanol-soluble oligosaccharides are now being added to foods as prebiotic ingredients, and fall within the category "dietary fiber" because of their putative benefits. Dietary fiber methods that measure 80% ethanol-soluble components are, therefore, now required for regulation.

In most plant foods oligosaccharides represent only a few per cent of NSP, but can be important in samples containing high levels of storage fructans, such as artichokes and onions (89). Furthermore, some higher DP (degree of polymerization) polysaccharides that appear to be of cell wall origin, including some arabinans and pectic polymers, have been found not to be fully recovered in standard fiber analyses that use 78–80% ethanol precipitation (45,46), which includes all methods shown in Table 3.

Where oligosaccharides are known to be present in foods, specific measurement of them is required to supplement the methods in Table 3. When the composition of a food is unknown, and it may contain oligosaccharides, a full analysis of the 80% ethanol-soluble carbohydrates will be required now that labeling requirements are linked to definitions of dietary fiber that include oligosaccharides. However, such instances are not likely to be common as most processed foods contain known ingredients.

E. Nondigestible Matter of Nonplant Origin

The latest definition of dietary fiber (3) stipulates that the nondigestible carbohydrates in dietary fiber must either be intrinsic to plant material or, if added to food as an ingredient, shown to be of physiological benefit to humans. Therefore, materials analyzing as dietary fiber in yeasts and other fungi, in bacteria, and in animal products must be excluded from fiber analysis, unless shown to be beneficial as added fiber. Such a division seems to be somewhat arbitrary, and not really consistent with the physiological concept of dietary fiber because events in the gut that affect physiology take place in response to the properties of the consumed nondigested molecules, and the gut makes no distinction according to the source of the dietary fiber.

Crustacean and insect polysaccharides such as chitin and chitosan, and animal polysaccharides such as chondroitin, are likely to precipitate in 80% ethanol and give a spuriously high dietary fiber result for any method based on a definition of dietary fiber that restricts it to nondigestible plant polysaccharides. If the exclusion of animal and nonplant polysaccharides is to be enforced there will need to be a good deal of methodological development to allow them to be distinguished from dietary fiber.

VI. SUPPLEMENTARY ANALYSES FOR EXTENDED DEFINITIONS OF DIETARY FIBER

The extended definition of dietary fiber as nondigestible carbohydrate and lignin intrinsic to plants, plus

extrinsic isolated nondigestible carbohydrates not of animal origin, demands additional measurements to exclude nondigestible carbohydrates of animal origin. Additional measurements are also required to account for both resistant starch and 80% ethanol-soluble carbohydrates that are not or are only partially measured in the current methods for dietary fiber analysis. The methods of Prosky, Englyst, and Theander are not made redundant by the need for new measurements, but can be used in conjunction with them to achieve the same goal—full accounting for the components of dietary fiber as defined, as follows (3):

1. Using gravimetric methods (AOAC methods 985.29, 991.43, 992.16, 993.21) or the method of Theander:
   a. Subtract resistant starch
   b. Add naturally occurring resistant starch
   c. Add naturally occurring oligosaccharides
   d. Subtract nondigestible, animal-derived carbohydrate
2. Using the methods of Englyst (colorimetric, GC, or HPLC):
   a. Add lignin
   b. Add naturally occurring resistant starch
   c. Add naturally occurring oligosaccharides
   d. Subtract nondigestible, animal-derived carbohydrate

In many foods the presence of nondigestible carbohydrates of animal origin would be very low.

Methods that can be applied to samples of unknown composition to distinguish adequately between nondigestible carbohydrates "intrinsic and intact" in plants, added fiber that qualifies as fiber only if it is of demonstrated physiological benefit, and fibers of nonplant origin is an enormous analytical challenge, in view of the polymolecular, polydisperse nature of food carbohydrates. Disentangling the fiber components in complex foods consisting of many ingredients, in order to define the fraction that might qualify as dietary fiber, will be difficult to achieve.

The procedures of Englyst and coworkers (35,53) are probably those that are most readily adapted to account for the components of dietary fiber. Their measurement of NSP, resistant starch, and monosaccharide components with separation of neutral sugars and hexosamines by HPLC (90), when supplemented with a lignin measurement may provide a reasonable measure of total dietary fiber. Englyst and coworkers have always maintained that resistant starch and lignin should be measured as discrete food components (48,49).

Recent developments in methodology have focused on accurate measurement of resistant starch, and oligosaccharides that are not precipitated by 80% ethanol. It should be noted, however, that most of the methods for oligosaccharide measurement have depended on knowing in advance the species of oligosaccharide present. Practical methods for accurate measurement of components specified in the latest definition of dietary fiber will require a good deal of further development, especially in the analysis of samples of unknown composition.

A. Resistant Starch

Several methods have been developed for measuring resistant starch (RS) (91). The general principle governing them all is the same, so most of them are quite similar, although they differ in detail.

1. Principle

Digestible starch is digested from the sample using α-amylase, and the undigested starch is either measured separately by gelatinizing it with 2 M KOH, enzymically digesting it, and measuring glucose released, or is measured as the difference between digested starch and total starch in the sample before digestion.

An outline of recent methods for RS analysis is presented below. The original publications should be consulted for full details.

2. Englyst et al. (53)

a. Sample Pretreatment. Minced using a 9 mm aperture plate.

b. Enzymes Used. Pancreatin, amyloglucosidase, invertase.

c. Sample Digestion. Sample (0.8–4.0 g) is digested in acetate buffer (pH 5.2, 37°C, 20 mL) with pancreatin/amyloglucosidase in 50 mL tubes with five 1.5 cm glass balls and horizontal shaking to mimick the mechanical effects of the gut, and guar gum to mimick gut viscosity. Samples are taken at 20 min for rapidly digestible starch (RDS) and 120 min for RDS plus slowly digested starch (SDS). Total starch (TS) is measured in a separate sample after dispersion with KOH and digestion, and free glucose is measured after invertase digestion and subtracted.

d. RS Separation. An RS containing residue is not separated.

e. RS Digestion. RS is not digested as RS measurement is by difference.

f. RS Measurement. Digested starch is measured as glucose in the digests by glucose oxidase, and RS calculated by difference [RS = TS − (RDS + SDS)].

g. Comment. A validated method that provides useful information on starch digestion in a range of foods, but if the aim is to measure total RS alone the method is unnecessarily involved. The method to measure RS_1, RS_2, and RS_3 separately is described in the same paper.

3. Muir and O'Dea (51,92)

a. Sample Pretreatment. Sample is chewed to the point of normal swallowing.

b. Enzymes Used. Pepsin, α-amylase, amyloglucosidase.

c. Sample Digestion. Sample containing about 0.1 g starch is suspended in 1 mL pepsin (pH 2, 37°C, 30 min) to mimick gastric conditions, and neutralized with NaOH, before adding 5 mL acetate buffer (0.2 M, pH 5.0), containing 10 mg α-amylase and 28 U amyloglucosidase, 15 h, 37°C.

d. RS Separation. Tubes are centrifuged (10 min, 1200 *g*), the supernatant removed, and the pellet washed with buffer.

e. RS Digestion. Resistant starch in the pellet is dispersed by boiling (20 min), followed by a 2 M KOH (0°C, 30 min) treatment with acetic acid, then added to pH 5, and the dispersed RS digested with amyloglucosidase.

f. RS Measurement. RS measured as glucose released using glucose oxidase (GOD–PAP).

f. Comment. Validated against human ileostomy model. Has not been widely used as a standardized method.

4. Goñi et al. (93)

a. Sample Pretreatment. Milled to 1 mm if dry or homogenized if wet.

b. Enzymes Used. Pepsin, pancreatic α-amylase, amyloglucosidase.

c. Sample Digestion. Pepsin (pH 1.5, 40°C, 60 min) followed by pancreatic α-amylase (pH 6.9, 37°C, 16 h).

d. RS Separation. RS in the digested residue is collected by centrifugation.

e. RS Digestion. RS dispersed in 2 M KOH (room temperature, 30 min), then digested with amyloglucosidase (60°C, 45 min) in acetate buffer.

f. RS Measurement. Enzymatic GOD–PAP measurement of glucose released.

g. Comment. Uses only pepsin and pancreatin in the digestion phase so avoids any overdigestion by amyloglucosidase. No ethanolic precipitation, so any soluble but nondepolymerized fragments may not be accounted for.

5. Åkerberg et al. (54)

a. Sample Pretreatment. Samples are chewed under standardized conditions (1 g, 15 times, 15 s).

b. Enzymes Used. Pepsin, pancreatin, amyloglucosidase.

c. Sample Digestion. Chewed sample (1 g) is expectorated into a beaker containing 5 mL water plus 1 mL pepsin (50 g /L, 2000FIB-U/g), the mouth rinsed with a further 5 mL water, which is added to the beaker, the pH adjusted to 1.5 with 2 M HCl, and the contents incubated 37°C, 30 min. Acetate buffer (10 mL, pH 5, 0.5 M) is added, the pH adjusted to 5.0, followed by addition of 125 μL $MgCl_2$ (0.06 M)–$CaCl_2$ (0.3 M), 125 μL pancreatin (40 g/L, 8 × USP), 400 μL amyloglucosidase (3500 U), 100 μL isopropanol, and water to final volume of 50 mL. The samples are then incubated with stirring 16 h, 40°C.

d. RS Separation. Four × the incubation volume of 95% ethanol is added and the precipitate collected on Celite in porosity 2 crucibles.

e. RS Digestion. A subsample of dried, RS filter residue is ground to release encapsulated starch and up to 0.5 g dispersed in 2 M KOH (30 min), acetic acid and acetate buffer are added before digesting with heat-stable amylase and amyloglucosidase.

f. RS Measurement. Enzymatic GOD–POD.

g. Comments. The method (details not presented here) can be used to measure total dietary fiber as NSP plus lignin from the weight of the RS filter residue

corrected for indigestible protein and ash. Validated against literature values for RS in ileostomy effluents.

6. Champ et al. (94)

a. Sample Pretreatment. Minced using a 9 mm aperture plate.

b. Enzymes Used. Pancreatic α-amylase, amyloglucosidase.

c. Sample Digestion. No pepsin digestion. Sample containing 50 mg starch is weighed into a 100 mL centrifuge tube, 5.8 mL Tris-maleate buffer (0.1 M, pH 5.25 containing 4 mM $CaCl_2$ and 0.02% sodium azide) is added plus 4 mL pancreatic α-amylase (10 mg/mL) and 0.2 mL amyloglucosidase (6 AGU/ml) and the sample digested 16 h, 37°C.

d. RS Separation. Ethanol (40 mL) is added, the tubes centrifuged 3000 *g*, 10 min, and pellet rinsed 2 × with 80% ethanol.

e. RS Digestion. The pellet is suspended in 10 mL water (100°C, 20 min), cooled to 0°C, 10 mL 4 M KOH at 0°C added, and the sample extracted 30 min 0°C with shaking. An aliquot (1 mL) of RS dispersion is added to 10 mL acetic acid (0.5 M, containing 4 mM $CaCl_2$), and 0.2 mL amyloglucosidase (14 AGU/ml) added, before incubating for 30 min, 70°C, then 10 min, 100°C, neutralizing with 0.6 mL 4 M KOH, and centrifuging 10 min, 4000 *g*.

f. RS Measurement. A 0.1 mL aliquot of RS digest is added to 2 mL GOD–PAP reagent to measure resistant starch as free glucose.

g. Comments. Straightforward assay. Does not use a pepsin digestion, and includes amyloglucosidase to reduce product inhibition of amylase. Predicts RS from ileostomates but underestimates data from intubation.

6. McLeary et al. (95)

a. Sample Pretreatment. Freeze-dried samples are milled to pass a 1.0 mm sieve, or fresh samples minced to pass a 4 mm screen.

b. Enzymes Used. Amyloglucosidase, pancreatic α-amylase.

c. Sample Digestion. Sample (100 mg) is weighed into screw-cap culture tubes, 4.0 mL of the centrifuged supernatant of pancreatic α-amylase (1 g amylase/100 mL Na maleate buffer, 0.1 M, pH 6.0, containing 0.3 g $CaCl_2$/L and 0.2 g Na azide/L) containing 3 U/mL amyloglucosidase is added, and the tubes incubated (37°C, 16 h) with shaking (200 strokes/min).

d. RS Separation. Industrial methylated spirits (99% v/v, 4 mL) is added, the tube contents mixed, and the tube centrifuged (1500 *g*). The pellet is resuspended 2 × in 50% methylated spirits and collected by centrifuging.

e. RS Digestion. The pellet is dispersed in 2 mL 2 M KOH (0°C, 20 min), 1.2 M sodium acetate buffer (8 mL) is added, the contents mixed, then 0.1 mL amyloglucosidase (3300 U/mL) added and the tube incubated 50°C, 30 min.

f. RS Measurement. After centrifuging (1500 *g*, 10 min) the RS digest, a 0.1 mL aliquot is used directly if the sample contains < 10% RS, or is diluted to 100 mL in volumetric flask if it contains > 10% RS, before removing a 0.1 mL aliquot into 3 mL GOD–POD reagent for glucose analysis.

g. Comments. This method is reproducible and straightforward but is best suited to materials in which the resistant starch component does not depend on food structure because the method is designed for use with finely milled samples.

B. Nondigestible Nonstarch Carbohydrates not Precipitated by 80% Ethanol

Methods to account for nondigestible carbohydrates that are not precipitated by 80% ethanol have largely depended on a priori knowledge of what there is to measure. That is, the methods have been designed to measure "added dietary fiber," which is necessarily of known identity if added as a food ingredient. The need to measure 80% ethanol-soluble fiber components has increased enormously in recent years with the demand for foods containing prebiotics such as fructo-oligosaccharides.

Where the aim has been to achieve dietary fiber analysis while accounting for 80% ethanol-soluble, nondigestible polysaccharides, the approach generally taken has been to conduct a usual analysis of dietary fiber, with 80% ethanol precipitation, and then to measure the materials of interest in the ethanolic extract. Therefore, measurement of 80% ethanol-soluble materials has become an add-on to total fiber analysis, as was the case in soluble fiber analysis.

However, when all of a material that is not fully recovered in usual fiber analysis is measured separately from fiber analysis, care has to be taken that all of the component is then excluded from the dietary fiber

analysis, or doubling up will occur, leading to an overestimation of dietary fiber.

To date, a limited number of supplementary procedures have been devised to measure a small number of indigestible oligosaccharide components of food. If analysis of 80% ethanol-soluble fiber components depends on specific tests, then as the list of such components extends, even more specific tests will be required. Analysis of samples of unknown composition could then become increasingly complex. A dietary fiber procedure that can measure all nondigestible food carbohydrate in one hit, without supplementary measurement of specific components, is not yet available for routine food analysis.

Dietary fiber components for which specific analyses have been developed to supplement the certified AOAC procedures for total dietary fiber include β-glucans, oligofructans and inulin, polydextrose, and galacto-oligosaccharides. Specific tests for such compounds require the use of specific enzymes of assured purity.

1. β-Glucan

Cereal β-glucan has received a good deal of attention for its hypocholesterolemic activity, both in oat and barley products, and in more concentrated forms such as oat gum. In the U.S.A. health claims associated with soluble fiber from oats have been permitted, making it important to be able to measure it accurately for food labeling.

The principle underlying methods for measuring β-glucan is that it is depolymerized with lichenase to β-oligosaccharides, and the β-oligosaccharides are then depolymerized with β-glucosidase, allowing measurement of the β-glucan as free glucose by the glucose oxidase method. Two AOAC methods are available for β-glucan determination, AOAC method 992.28 for (1–3)(1–4)-β-D-glucans in oat and barley fractions and ready-to-eat cereals, and the AACC–AOAC method 995.16, the "streamlined" enzymatic method for β-D-glucans in barley, oats, and cereal products (96). The latter method is outlined below.

a. Sample Pretreatment. Sample is finely milled using a 0.5 mm sieve, or is cyclone milled. Samples containing free glucose should be pre-extracted with 80% ethanol (10 mL, 80°C, 2 ×) and collected by centrifugation.

b. Enzymes Used. Lichenase (50 U/mL in 0.02 M phosphate buffer, pH 6.5), β-glucosidase (2 U/mL in 50 mM acetate buffer, pH 4.0).

c. Sample Digestion. Sample (80–100 mg) is accurately weighed into a 17 mL tube, pre-extracted with 80% ethanol to remove glucose if necessary (*a*, above), 0.2 mL 50% ethanol added, the sample stirred, and 4.0 mL Na phosphate buffer (0.02 M, pH 6.5) added. The sample is dispersed, heated (100°C, 1 min) vortex stirred, reheated (100°C, 2 min), again vortex stirred, and the tubes placed in a 50°C water bath. Lichenase solution (0.2 mL) is added, and the tubes incubated (60 min, 50°C), with mixing several times. Na acetate buffer (5 mL, 0.2 M, pH 4.0) is added, mixed, and cooled to room temperature, before centrifuging (1000 *g*, 10 min) and removing 0.1 mL aliquots of supernatant to glass tubes (triplicate). To two of the tubes, 0.1 mL β-glucosidase (2 U/mL) solution is added, and the tube incubated 50°C, 10 min. Add 0.1 mL 0.05 M acetate buffer to the third tube as a blank.

d. Measurement. Add 3.0 mL GOD–POD reagent to measure glucose colorimetrically.

e. Comments. Because the measurement of β-glucan often takes place against a background of starch and cellulose, absolute specificity and freedom from contamination of the lichenase and β-glucosidase preparations are necessary. Appropriate indicative controls, including starch controls, should be taken through the procedure.

2. Fructo-oligosaccharides and Fructans

Fructo-oligosaccharides (DP < 9), and fructans including inulin, are now big business as prebiotics, fat replacers, and dietary fiber ingredients. They have been accepted as dietary fiber based on their nondigestible carbohydrate nature and physiological effects, such as prebiotic activity and mild laxation (97). They are not fully recovered during fiber analysis involving 80% ethanol precipitation, but for regulatory purposes measurement of fructans and fructo-oligosaccharides is important.

Several methods exist for measuring fructo-oligosaccharides and fructans in foods, and they are all enzymatic, involving hydrolysis to fructose and glucose, but differ in the way the fructan-derived sugars are analytically distinguished from others present. In AOAC Method 997.08, chromatography is used after specific enzyme digestion to separate fructan, sucrose, glucose, and starch (98). Dionex ion chromatography after invertase hydrolysis has provided a specific analysis of short-chain fructo-oligosaccharides (99). In AOAC Method 999.03, purified enzymes (sucrase, β-amylase, pullulanase, maltase) are used to

depolymerize completely nonfructan-available carbohydrates to monosaccharides, which are reduced. Fructan is then measured as the amount of reducing sugar that is restored on *exo*-inulinase plus *endo*-inulinase treatment, measured with the PAHBAH colorimetric method for reducing sugars (Table 5), which gives the same response for glucose and fructose (100).

AOAC Method 999.03 is not demanding in terms of equipment and techniques, so is suited to routine food laboratory analyses run in conjunction with standard dietary fiber analysis. It does require highly purified enzymes used at the right concentrations, and these are available in kit form (Megazyme; Code: K-FRUC). The method has shown good reliability in interlaboratory evaluations (100).

3. Galacto-oligosaccharides

Galacto-oligosaccharides are not recovered in dietary fiber analysis, but, because they are nondigestible carbohydrates with the supposedly beneficial prebiotic effect of increasing bifidobacteria in the colon, they qualify as dietary fiber and require measurement. They are available in two forms that require slightly different treatments in analysis, galactosylsucrose oligosaccharides from plants, and a galactosylated glucose series produced by galactosylation of glucose by β-galactosidase.

Measurement of galactosylsucrose oligosaccharides employs total hydrolysis by α-galactosidase, and the galactosylglucose assay uses β-galactosidase. There is a requirement for highly specific enzyme activity if the release of monosaccharides is to be attributed solely to the presence of galacto-oligosaccharides. The monosaccharides released can be measured chromatographically or by colorimetry (7).

4. Polydextrose

Polydextrose is a synthetic glucose-based polysaccharide that is used as a bulking agent in some food products. It is prepared by polymerizing glucose with sorbitol and citric acid. Amongst the bonds formed are some that link ascorbic acid to the polymer, giving it a charge that is made use of in analyzing polydextrose. It is nondigestible and has prebiotic effects that qualify it for definition as dietary fiber, increasing *Lactobacillus* and *Bifidobacterium* species in the colon and leading to an increase in butyrate production.

A method for measuring polydextrose has been developed and approved of by AOAC. It involves water extraction with centrifugal ultrafiltration to remove high molecular weight solubles, multienzyme hydrolysis to depolymerize all digestible carbohydrates, and finally, taking advantage of the charged nature of polydextrose, anion-exchange chromatography with electrochemical detection (101). The method showed reasonable repeatability and reproducibility in a collaborative study (102).

5. Resistant Maltodextrins

Measurement of digestion-resistant maltodextrins is part of a recently published method for measuring total dietary fiber in foods containing maltodextrins. The approach taken essentially involves measuring TDF by method AOAC 985.29 or AOAC 991.43, involving 78% ethanol precipitation and fiber recovery by filtration, but with additional measurement of maltodextrin in the combined ethanolic filtrate and acetone wash. Maltodextrin was measured by HPLC after removing salts and proteins with anion-exchange resins.

A collaborative study of the method when used to measure total dietary fiber as the sum of insoluble fiber plus high molecular weight soluble fiber plus resistant maltodextrin, showed good reproducibility and repeatability (103).

VII. INTERPRETING DIETARY FIBER RESULTS

The methods for dietary fiber analysis discussed here give information solely about the quantity of dietary fiber in a food. They do not provide information about polysaccharide properties—degrees of polymerization, distribution of side chains, cross-links, and so on—that govern physical properties of fiber, and in turn, physiological responses to it. They give no clues to cell wall, cell, or plant tissue morphology, all of which contribute to the effects of fiber in the gut. Emergent properties of the organized systems of cell wall polymers and cells interacting with the gut, such as coarseness or resistance to bacteria, or fecal bulking efficacy, cannot be accessed by dietary fiber analyses alone. Similarly, a measurement of soluble fiber will not indicate the impact on gut viscosity.

It is expecting too much of fiber analysis that it should provide a sure basis for predicting effects on health, which depend on much more than mere quantity of fiber consumed. Fiber analyses are similar to most other analytical procedures in this respect.

If effects on physiological states are to be predicted, a range of physicochemical properties such as viscosity, water-holding capacity, particle size, fermentability, and responses to them must be measured. Solubility is the only physical variable that is measured in fiber analysis, but, even so, the conditions of solubilization have seldom been physiological.

Despite the important place of physiological actions in the definition and analysis of dietary fiber, dietary fiber analyses are not physiological—they merely use enzymes to achieve a level of specificity that reflects the specificity of gut enzymes, and they exclude a large amount of undigested matter that is not carbohydrate or lignin. The physiological effects of foods and of food remains in the gut are an expression of the complex interaction of all of the remains of food with one another, with the gut, and with gut bacteria. That is why dietary fiber content is not a reliable predictor, even of such classical dietary fiber effects as fecal bulking (104). A definition of dietary fiber that is restricted to indigestible carbohydrates and lignin is unlikely to reflect the true physiological impact of nondigestible material in the gut.

The definition of dietary fiber as "Any dietary component that reaches the colon without being absorbed in a healthy human gut" (14) is a more realistic definition in terms of colonic loading, although it is one that creates analytical problems if analysis is to be based on the sum of components measured specifically, and if one wishes to identify mechanisms in disease. Similarly, gravimetric measurement of the fraction of foods not digested under physiological conditions is likely to provide a nutritionally more valid index of the colonic loading imposed by a food than a measurement that restricts itself to a few food components. An in vitro determination of the indigestible fraction of foods has recently been published as an alternative to dietary fiber analysis (105).

Dietary fiber analysis for the purposes of food labeling is intended to inform the public about the amount of a functional food component present in a food. However, the only stipulation in dietary fiber definitions, with respect to physiological effects, is that there be some benefit. There is no way that a dietary fiber value can inform a consumer of the type of benefit or the degree of benefit or function that the fiber value represents (106). The physiological effects of dietary fiber are too diverse to be represented by a single datum, so in terms of informing the public about benefits, a dietary fiber value on a food label is not very useful at all. Some dietary fibers may lower blood cholesterol with little effect on fecal bulk, others are good laxatives that have no effect on blood cholesterol, yet others can do both, and others will do neither to a useful extent.

In general, therefore, the relationship between dietary fiber content of a food or diet and health may be difficult to interpret if one relies solely on dietary fiber values. Instead, it is best that defined components are measured because they are often linked to health through mechanisms that are specific to the food component, and, when relevant food components have been identified, a dietary fiber value may not be useful.

VIII. THE FUTURE

The future for dietary fiber is difficult to predict, as there remain inconsistencies and practical problems with the way it is being defined. As even more components are included in the definition of dietary fiber, it is likely to become increasingly difficult to analyze. The distinction between dietary fiber and other nondigestible food components is quite arbitrary, although historically it is linked to the original concept of plant cell walls in the diet. However, in reality, the undigested fraction of foods can contain all of the food components that are present in the digested fraction if the physical nature of the food as consumed prevents digestion. The decision to select carbohydrates and lignin alone from amongst the population of undigested materials is not logical, even if it is historical.

Furthermore, as the amount of dietary fiber measured as undigested carbohydrate and lignin gives little indication of physiological impact, the physiological benefits are not easily inferred from a dietary fiber value. Perhaps, ultimately, it makes most sense to define dietary fiber globally as the undigested fraction of foods, as Ha et al. have proposed (14), and to measure it globally as the indigestible fraction of food, as Saura-Calixto et al. have suggested (105). Beyond such measurements, if a specific food component is thought to be having an effect, it can be measured specifically, in association with its specific effect. Then, a connection between a food component and health may be made, and the term dietary fiber could be dispensed with altogether.

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