ANALYSIS OF FOOD PRODUCTS

1. 1. Introduction

Food analysis is the discipline dealing with the development, application and study of analytical procedures for characterizing the properties of foods and their constituents. These analytical procedures are used to provide information about a wide variety of different characteristics of foods, including their composition, structure, physicochemical properties and sensory attributes. This information is critical to our rational understanding of the factors that determine the properties of foods, as well as to our ability to economically produce foods that are consistently safe, nutritious and desirable and for consumers to make informed choices about their diet. The objective of this course is to review the basic principles of the analytical procedures commonly used to analyze foods and to discuss their application to specific food components, *e.g.* lipids, proteins, water, carbohydrates and minerals. The following questions will be addressed in this introductory section: Who analyzes foods? Why do they analyze foods? What types of properties are measured? How does one choose an appropriate analytical technique for a particular food?

1.1. Reasons for Analyzing Foods

Foods are analyzed by scientists working in all of the major sectors of the food industry including food manufacturers, ingredient suppliers, analytical service laboratories, government laboratories, and University research laboratories. The various purposes that foods are analyzed are briefly discussed in this section.

1.1.1. Government Regulations and Recommendations

Government regulations and recommendations are designed to maintain the general quality of the food supply, to ensure the food industry provides consumers with foods that are wholesome and safe, to inform consumers about the nutritional composition of foods so that they can make knowledgeable choices about their diet, to enable fair competition amongst food companies, and to eliminate economic fraud. There are a number of Government Departments Responsible for regulating the composition and

quality of foods, including the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), the National Marine Fisheries Service (NMFS) and the Environmental Protection Agency (EPA). Each of these government agencies is responsible for regulating particular sectors of the food industry and publishes documents that contain detailed information about the regulations and recommendations pertaining to the foods produced within those sectors. These documents can be purchased from the government or obtained on-line from the appropriate website.

Standards

Government agencies have specified a number of voluntary and mandatory standards concerning the composition, quality, inspection, and labeling of specific food products.

Mandatory Standards:

- *Standards of Identity.* These regulations specify the type and amounts of ingredients that certain foods must contain if they are to be called by a particular name on the food label. For some foods there is a maximum or minimum concentration of a certain component that they must contain, *e.g.,* "peanut butter" must be less than 55% fat, "ice-cream" must be greater than 10% milk fat, "cheddar cheese" must be greater than 50% milk fat and less than 39% moisture.
- *Standards of Quality.* Standards of quality have been defined for certain foods (*e.g.,* canned fruits and vegetables) to set minimum requirements on the color, tenderness, mass and freedom from defects.
- *Standards of Fill-of-Container*. These standards state how full a container must be to avoid consumer deception, as well as specifying how the degree of fill is measured.

Voluntary Standards:

- *Standards of Grade.* A number of foods, including meat, dairy products and eggs, are *graded* according to their quality, *e.g.* from standard to excellent. For example meats can be graded as "prime", "choice", "select", "standard" etc according to their origin, tenderness, juiciness, flavor and appearance. There are clear definitions associated with these descriptors that products must conform to before they can be given the appropriate label. Specification of the grade of a food product on the label is voluntary, but many food manufacturers opt to do this because superior grade products can be sold for a higher price. The government has laboratories that food producers send their products too to be

tested to receive the appropriate certification. This service is requested and paid for by the food producer.

Nutritional Labeling

In 1990, the US government passed the Nutritional Labeling and Education Act (NLEA), which revised the regulations pertaining to the nutritional labeling of foods, and made it mandatory for almost all food products to have standardized nutritional labels. One of the major reasons for introducing these regulations was so that consumers could make informed choices about their diet. Nutritional labels state the total calorific value of the food, as well as total fat, saturated fat, cholesterol, sodium, carbohydrate, dietary fiber, sugars, protein, vitamins, calcium and iron. The label may also contain information about nutrient content claims (such as "low fat", "low sodium" "high fiber" "fat free" etc), although government regulations stipulate the minimum or maximum amounts of specific food components that a food must contain if it is to be given one of these nutrient content descriptors. The label may also contain certain FDA approved health claims based on links between specific food components and certain diseases (*e.g.*, calcium and osteoporosis, sodium and high blood pressure, soluble fiber and heart disease, and cholesterol and heart disease). The information provided on the label can be used by consumers to plan a nutritious and balanced diet, to avoid over consumption of food components linked with health problems, and to encourage greater consumption of foods that are beneficial to health.

Authenticity

The price of certain foods is dictated by the quality of the ingredients that they contain. For example, a packet of premium coffee may claim that the coffee beans are from Columbia, or the label of an expensive wine may claim that it was produced in a certain region, using a certain type of grapes in a particular year. How do we verify these claims? There are many instances in the past where manufacturers have made false claims about the authenticity of their products in order to get a higher price. It is therefore important to have analytical techniques that can be used to test the authenticity of certain food components, to ensure that consumers are not the victims of economic fraud and that competition among food manufacturers is fair.

Food Inspection and Grading

The government has a *Food Inspection and Grading Service* that routinely analyses the properties of food products to ensure that they meet the appropriate laws and regulations. Hence, both government agencies and food manufacturers need analytical techniques to provide the appropriate information about food properties. The most important criteria for this type of test are often the accuracy of the measurements and the use of an official method. The government has recently carried out a survey of many of the official analytical techniques developed to analyze foods, and has specified which techniques must be used to analyze certain food components for labeling purposes. Techniques have been chosen which provide accurate and reliable results, but which are relatively simple and inexpensive to perform.

1.1.2. Food Safety

One of the most important reasons for analyzing foods from both the consumers and the manufacturers standpoint is to ensure that they are safe. It would be economically disastrous, as well as being rather unpleasant to consumers, if a food manufacturer sold a product that was harmful or toxic. A food may be considered to be unsafe because it contains harmful microorganisms (*e.g.*, Listeria, Salmonella), toxic chemicals (*e.g.*, pesticides, herbicides) or extraneous matter (*e.g.*, glass, wood, metal, insect matter). It is therefore important that food manufacturers do everything they can to ensure that these harmful substances are not present, or that they are effectively eliminated before the food is consumed. This can be achieved by following "good manufacturing practice" regulations specified by the government for specific food products and by having analytical techniques that are capable of detecting harmful substances. In many situations it is important to use analytical techniques that have a high sensitivity, *i.e.*, that can reliably detect low levels of harmful material. Food manufacturers and government laboratories routinely analyze food products to ensure that they do not contain harmful substances and that the food production facility is operating correctly.

1.1.3. Quality control

The food industry is highly competitive and food manufacturers are continually trying to increase their market-share and profits. To do this they must ensure that their products are of *higher quality, less expensive*, and more *desirable* than their competitors, whilst ensuring that they are *safe* and *nutritious.* To meet these rigorous standards food manufacturers need analytical techniques to analyze food materials before, during and after the manufacturing process

to ensure that the final product meets the desired standards. In a food factory one starts with a number of different raw materials, processes them in a certain manner (*e.g.* heat, cool, mix, dry), packages them for consumption and then stores them. The food is then transported to a warehouse or retailer where it is sold for consumption.

One of the most important concerns of the food manufacturer is to produce a final product that consistently has the same overall properties, *i.e.* appearance, texture, flavor and shelf life. When we purchase a particular food product we expect its properties to be the same (or very similar) to previous times, and not to vary from purchase-to-purchase. Ideally, a food manufacture wants to take the raw ingredients, process them in a certain way and produce a product with specific desirable properties. Unfortunately, the properties of the raw ingredients and the processing conditions vary from time to time which causes the properties of the final product to vary, often in an unpredictable way. How can food manufacturers control these variations? Firstly, they can understand the role that different food ingredients and processing operations play in determining the final properties of foods, so that they can rationally control the manufacturing process to produce a final product with consistent properties. This type of information can be established through research and development work (see later). Secondly, they can monitor the properties of foods during production to ensure that they are meeting the specified requirements, and if a problem is detected during the production process, appropriate actions can be taken to maintain final product quality.

Characterization of raw materials. Manufacturers measure the properties of incoming raw materials to ensure that they meet certain minimum standards of quality that have previously been defined by the manufacturer. If these standards are not met the manufacturer rejects the material. Even when a batch of raw materials has been accepted, variations in its properties might lead to changes in the properties of the final product. By analyzing the raw materials it is often possible to predict their subsequent behavior during processing so that the processing conditions can be altered to produce a final product with the desired properties. For example, the color of potato chips depends on the concentration of reducing sugars in the potatoes that they are manufactured from: the higher the concentration, the browner the potato chip. Thus it is necessary to have an analytical technique to measure the concentration of reducing sugars in the potatoes so that the frying conditions can be altered to produce the optimum colored potato chip.

Monitoring of food properties during processing. It is advantageous for food manufacturers to be able to measure the properties of foods during processing. Thus, if any problem develops, then it can be quickly detected, and the process adjusted to compensate for it. This helps to improve the overall quality of a food and to reduce the amount of material and time wasted. For example, if a manufacturer were producing a salad dressing product, and the oil content became too high or too low they would want to adjust the processing conditions to eliminate this problem. Traditionally, samples are removed from the process and tested in a quality assurance laboratory. This procedure is often fairly time-consuming and means that some of the product is usually wasted before a particular problem becomes apparent. For this reason, there is an increasing tendency in the food industry to use analytical techniques which are capable of rapidly measuring the properties of foods on-line, without having to remove a sample from the process. These techniques allow problems to be determined much more quickly and therefore lead to improved product quality and less waste. The ideal criteria for an on-line technique is that it be capable of rapid and precise measurements, it is non-intrusive, it is nondestructive and that it can be automated.

Characterization of final product. Once the product has been made it is important to analyze its properties to ensure that it meets the appropriate legal and labeling requirements, that it is safe, and that it is of high quality. It is also important to ensure that it retains its desirable properties up to the time when it is consumed.

A system known as **Hazard Analysis and Critical Control Point** (HACCP) has been developed, whose aim is to systematically identify the ingredients or processes that may cause problems (hazard analysis), assign locations (critical control points) within the manufacturing process where the properties of the food must be measured to ensure that safety and quality are maintained, and to specify the appropriate action to take if a problem is identified. The type of analytical technique required to carry out the analysis is often specified. In addition, the manufacturer must keep detailed documentation of the performance and results of these tests. HACCP was initially developed for safety testing of foods, but it or similar systems are also now being used to test food quality.

1.1.4. Research and Development

In recent years, there have been significant changes in the preferences of consumers for foods that are healthier, higher

quality, lower cost and more exotic. Individual food manufacturers must respond rapidly to these changes in order to remain competitive within the food industry. To meet these demands food manufacturers often employ a number of scientists whose primary objective is to carry out research that will lead to the development of new products, the improvement of existing products and the reduction of manufacturing costs.

Many scientists working in universities, government research laboratories and large food companies carry out *basic research*. Experiments are designed to provide information that leads to a better understanding of the role that different ingredients and processing operations play in determining the overall properties of foods. Research is mainly directed towards investigating the structure and interaction of food ingredients, and how they are effected by changes in environment, such as temperature, pressure and mechanical agitation. Basic research tends to be carried out on simple model systems with well-defined compositions and properties, rather than real foods with complex compositions and structures, so that the researchers can focus on particular aspects of the system. Scientists working for food companies or ingredient suppliers usually carry out *product development*. Food Scientists working in this area use their knowledge of food ingredients and processing operations to improve the properties of existing products or to develop new products. In practice, there is a great deal of overlap between basic research and product development, with the basic researchers providing information that can be used by the product developers to rationally optimize food composition and properties. In both fundamental research and product development analytical techniques are needed to characterize the overall properties of foods (*e.g.*, color, texture, flavor, shelf-life *etc.*), to ascertain the role that each ingredient plays in determining the overall properties of foods, and to determine how the properties of foods are affected by various processing conditions (*e.g.*, storage, heating, mixing, freezing).

1.2 Properties Analyzed

Food analysts are interested in obtaining information about a variety of different characteristics of foods, including their composition, structure, physicochemical properties and sensory attributes.

1.2.1 Composition

The composition of a food largely determines its safety, nutrition, physicochemical properties, quality attributes and sensory characteristics. Most foods are compositionally complex materials made up of a wide variety of different chemical constituents. Their composition can be specified in a number of different ways depending on the property that is of interest to the analyst and the type of analytical procedure used: specific atoms (*e.g.*, Carbon, Hydrogen, Oxygen, Nitrogen, Sulfur, Sodium, etc.); specific molecules (*e.g.*, water, sucrose, tristearin, β–lactoglobulin), types of molecules (*e.g.*, fats, proteins, carbohydrates, fiber, minerals), or specific substances (*e.g.*, peas, flour, milk, peanuts, butter). Government regulations state that the concentration of certain food components must be stipulated on the nutritional label of most food products, and are usually reported as specific molecules (*e.g.*, vitamin A) or types of molecules (*e.g.*, proteins).

1.2.2 Structure

The structural organization of the components within a food also plays a large role in determining the physicochemical properties, quality attributes and sensory characteristics of many foods. Hence, two foods that have the same composition can have very different quality attributes if their constituents are organized differently. For example, a carton of ice cream taken from a refrigerator has a pleasant appearance and good taste, but if it is allowed to melt and then is placed back in the refrigerator its appearance and texture change dramatically and it would not be acceptable to a consumer. Thus, there has been an adverse influence on its quality, even though its chemical composition is unchanged, because of an alteration in the structural organization of the constituents caused by the melting of ice and fat crystals. Another familiar example is the change in egg white from a transparent viscous liquid to an optically opaque gel when it is heated in boiling water for a few minutes. Again there is no change in the chemical composition of the food, but its physiochemical properties have changed dramatically because of an alteration in the structural organization of the constituents caused by protein unfolding and gelation.

The structure of a food can be examined at a number of different levels:

- - *Molecular structure* (~ 1 – 100 nm). Ultimately, the overall physicochemical properties of a food depend on the type of molecules present, their three-dimensional structure and their interactions with each other. It is therefore important

for food scientists to have analytical techniques to examine the structure and interactions of individual food molecules.

- *Microscopic structure* (~ 10 nm – 100 μm). The microscopic structure of a food can be observed by microscopy (but not by the unaided eye) and consists of regions in a material where the molecules associate to form discrete phases, *e.g.*, emulsion droplets, fat crystals, protein aggregates and small air cells.
- *Macroscopic structure* (~ > 100 μm). This is the structure that can be observed by the unaided human eye, *e.g.*, sugar granules, large air cells, raisons, chocolate chips.

The forgoing discussion has highlighted a number of different levels of structure that are important in foods. All of these different levels of structure contribute to the overall properties of foods, such as texture, appearance, stability and taste. In order to design new foods, or to improve the properties of existing foods, it is extremely useful to understand the relationship between the structural properties of foods and their bulk properties. Analytical techniques are therefore needed to characterize these different levels of structure. A number of the most important of these techniques are considered in this course.

1.2.3. Physicochemical Properties

The physiochemical properties of foods (rheological, optical, stability, "flavor") ultimately determine their perceived quality, sensory attributes and behavior during production, storage and consumption.

- The *optical properties* of foods are determined by the way that they interact with electromagnetic radiation in the visible region of the spectrum, *e.g.*, absorption, scattering, transmission and reflection of light. For example, full fat milk has a "whiter" appearance than skim milk because a greater fraction of the light incident upon the surface of full fat milk is scattered due to the presence of the fat droplets.
- The *rheological properties* of foods are determined by the way that the shape of the food changes, or the way that the food flows, in response to some applied force. For example, margarine should be spreadable when it comes out of a refrigerator, but it must not be so soft that it collapses under its own weight when it is left on a table.
- The *stability* of a food is a measure of its ability to resist changes in its properties over time. These changes may be chemical, physical or biological in origin. *Chemical stability* refers to the change in the type of molecules present in a food with time due to chemical or biochemical reactions, *e.g.*, fat

rancidity or non-enzymatic browning. *Physical stability* refers to the change in the spatial distribution of the molecules present in a food with time due to movement of molecules from one location to another, *e.g.*, droplet creaming in milk. *Biological stability* refers to the change in the number of microorganisms present in a food with time, *e.g.*, bacterial or fungal growth.

- The *flavor* of a food is determined by the way that certain molecules in the food interact with receptors in the mouth (taste) and nose (smell) of human beings. The perceived flavor of a food product depends on the type and concentration of flavor constituents within it, the nature of the food matrix, as well as how quickly the flavor molecules can move from the food to the sensors in the mouth and nose. Analytically, the flavor of a food is often characterized by measuring the concentration, type and release of flavor molecules within a food or in the headspace above the food.

Foods must therefore be carefully designed so that they have the required physicochemical properties over the range of environmental conditions that they will experience during processing, storage and consumption, *e.g.*, variations in temperature or mechanical stress. Consequently, analytical techniques are needed to test foods to ensure that they have the appropriate physicochemical properties.

1.2.4. Sensory Attributes

Ultimately, the quality and desirability of a food product is determined by its interaction with the sensory organs of human beings, *e.g.*, vision, taste, smell, feel and hearing. For this reason the sensory properties of new or improved foods are usually tested by human beings to ensure that they have acceptable and desirable properties before they are launched onto the market. Even so, individuals' perceptions of sensory attributes are often fairly subjective, being influenced by such factors as current trends, nutritional education, climate, age, health, and social, cultural and religious patterns. To minimize the effects of such factors a number of procedures have been developed to obtain statistically relevant information. For example, foods are often tested on statistically large groups of untrained consumers to determine their reaction to a new or improved product before full-scale marketing or further development. Alternatively, selected individuals may be trained so that they can reliably detect small differences in specific qualities of particular food products, *e.g.*, the mint flavor of a chewing gum.

Although sensory analysis is often the ultimate test for the acceptance or rejection of a particular food product, there are a

number of disadvantages: it is time consuming and expensive to carry out, tests are not objective, it cannot be used on materials that contain poisons or toxins, and it cannot be used to provide information about the safety, composition or nutritional value of a food. For these reasons objective analytical tests, which can be performed in a laboratory using standardized equipment and procedures, are often preferred for testing food product properties that are related to specific sensory attributes. For this reason, many attempts have been made to correlate sensory attributes (such as chewiness, tenderness, or stickiness) to quantities that can be measured using objective analytical techniques, with varying degrees of success.

1.3. Choosing an Analytical Technique

There are usually a number of different analytical techniques available to determine a particular property of a food material. It is therefore necessary to select the most appropriate technique for the specific application. The analytical technique selected depends on the property to be measured, the type of food to be analyzed, and the reason for carrying out the analysis. Information about the various analytical procedures available can be obtained from a number of different sources. An analytical procedure may already be routinely used in the laboratory or company where you are working. Alternatively, it may be possible to contact an expert who could recommend a certain technique, *e.g.,* a University Professor or a Consultant. Often it is necessary to consult scientific and technical publications. There are a number of different sources where information about the techniques used to analyze foods can be obtained:

1.3.1 Books

Food analysis books may provide a general overview of the various analytical procedures used to analyze food properties or they may deal with specific food components or physicochemical characteristics. Consulting a general textbook on food analysis is usually the best place to begin to obtain an overview of the types of analytical procedures available for analyzing foods and to critically determine their relative advantages and disadvantages.

Food Analysis, 2nd Edition. S.S. Nielsen, Aspen Publishers

Food Analysis: Theory and Practice. Y. Pomeranz & C.E. Meloan, Chapman and Hall

Food Analysis: Principles and Techniques. D.W. Gruenwedel and J.R. Whitaker, Marcel Dekker

Analytical Chemistry of Foods. C.S. James, Blackie Academic and Professional

1.3.2. Tabulated Official Methods of Analysis

A number of scientific organizations have been setup to establish certain techniques as official methods, *e.g.* Association of the Official Analytical Chemists (AOAC) and American Oil Chemists Society (AOCS). Normally, a particular laboratory develops a new analytical procedure and proposes it as a new official method to one of the organizations. The method is then tested by a number of independent laboratories using the same analytical procedure and type of equipment stipulated in the original proposal. The results of these tests are collated and compared with expected values to ensure that the method gives reproducible and accurate results. After rigorous testing the procedure may be accepted, modified or rejected as an official method. Organizations publish volumes that contain the officially recognized test methods for a variety of different food components and foodstuffs. It is possible to consult one of these official publications and ascertain whether a suitable analytical procedure already exists or can be modified for your particular application.

1.3.3. Journals

Analytical methods developed by other scientists are often reported in scientific journals, *e.g.*, Journal of Food Science, Journal of Agriculture and Food Chemistry, Journal of the American Oil Chemists Society, Analytical Chemistry. Information about analytical methods in journals can often be obtained by searching computer databases of scientific publications available at libraries or on the Internet (*e.g.*, Web of Science, Medline).

1.3.4. Equipment and Reagent Suppliers

Many companies that manufacture equipment and reagents used to analyze foods advertise their products in scientific journals, trade journals, trade directories, and the Internet. These companies will send you literature that describes the principles and specifications of the equipment or test procedures that they are selling, which can be used to determine the advantages and limitations of each technique.

1.3.5. Internet

The Internet is an excellent source of information on the various analytical procedures available for analyzing food properties. University lecturers, book suppliers, scientific organizations, scientific journals, computer databases, and equipment and reagent suppliers post information on the web about food analysis techniques. This information can be accessed using appropriately selected keywords in an Internet search engine.

1.3.6. Developing a New Technique

In some cases there may be no suitable techniques available and so it is necessary to develop a new one. This must be done with great care so as to ensure that the technique gives accurate and reliable measurements. Confidence in the accuracy of the technique can be obtained by analyzing samples of known properties or by comparing the results of the new technique with those of well-established or official methods.

One of the most important factors that must be considered when developing a new analytical technique is the way in which “the analyte” will be distinguished from “the matrix”. Most foods contain a large number of different components, and therefore it is often necessary to distinguish the component being analyzed for ("the analyte") from the multitude of other components surrounding it ("the matrix"). Food components can be distinguished from each other according to differences in their molecular characteristics, physical properties and chemical reactions:

- *Molecular characteristics:* Size, shape, polarity, electrical charge, interactions with radiation.
- *Physical properties:* Density, rheology, optical properties, electrical properties, phase transitions (melting point, boiling point).
- *Chemical reactions:* Specific chemical reactions between the component of interest and an added reagent.

When developing an appropriate analytical technique that is specific for a particular component it is necessary to identify the molecular and physicochemical properties of the analyte that are sufficiently different from those of the components in the matrix. In some foods it is possible to directly determine the analyte within the food matrix, but more often it is necessary to carry out a number of preparatory steps to isolate the analyte prior to carrying out the analysis. For example, an analyte may be physically isolated from the matrix using one procedure and then analyzed using another procedure. In some situations there may be one or more components within a food

that have very similar properties to the analyte. These "interferents" may make it difficult to develop an analytical technique that is specific for the analyte. It may be necessary to remove these interfering substances prior to carrying out the analysis for the analyte, or to use an analytical procedure that can distinguish between substances with similar properties.

1.4. Selecting an Appropriate Technique

Some of the criteria that are important in selecting a technique are listed below:

Precision: A measure of the ability to reproduce an answer between determinations performed by the same scientist (or group of scientists) using the same equipment and experimental approach.

Reproducibility: A measure of the ability to reproduce an answer by scientists using the same experimental approach but in different laboratories using different equipment.

Accuracy: A measure of how close one can actually measure the *true value* of the parameter being measured, *e.g.*, fat content, or sodium concentration.

Simplicity of operation: A measure of the ease with which relatively unskilled workers may carry out the analysis.

Cost: The total cost of the analysis, including the reagents, instrumentation and salary of personnel required to carry it out.

Speed: The time needed to complete the analysis of a single sample or the number of samples that can be analyzed in a given time.

Sensitivity: A measure of the lowest concentration of a component that can be detected by a given procedure.

Specificity: A measure of the ability to detect and quantify specific components within a food material, even in the presence of other similar components, *e.g.*, fructose in the presence of sucrose or glucose.

Safety: Many reagents and procedures used in food analysis are potentially hazardous *e.g.* strong acids or bases, toxic chemicals or flammable materials.

Destructive/Nondestructive: In some analytical methods the sample is destroyed during the analysis, whereas in others it remains intact.

On-line/Off-line: Some analytical methods can be used to measure the properties of a food during processing, whereas others can only be used after the sample has been taken from the production line.

Official Approval: Various international bodies have given official approval to methods that have been comprehensively studied by independent analysts and shown to be acceptable to the various organizations involved, *e.g.*, ISO, AOAC, AOCS.

Nature of Food Matrix: The composition, structure and physical properties of the matrix material surrounding the analyte often influences the type of method that can be used to carry out an analysis, *e.g.*, whether the matrix is solid or liquid, transparent or opaque, polar or non-polar.

If there are a number of alternative methods available for measuring a certain property of a food, the choice of a particular method will depend on which of the above criteria is most important. For example, *accuracy* and use of an *official method* may be the most important criteria in a government laboratory which checks the validity of compositional or nutritional claims on food products, whereas *speed* and the ability to make *nondestructive* measurements may be more important for routine quality control in a factory where a large number of samples have to be analyzed rapidly.

2. SAMPLING AND DATA ANALYSIS

2.1 Introduction

Analysis of the properties of a food material depends on the successful completion of a number of different steps: planning (identifying the most appropriate analytical procedure), sample selection, sample preparation, performance of analytical procedure, statistical analysis of measurements, and data reporting. Most of the subsequent chapters deal with the description of various analytical procedures developed to provide information about food properties, whereas this chapter focuses on the other aspects of food analysis.

2.2 Sample Selection and Sampling Plans

A food analyst often has to determine the characteristics of a large quantity of food material, such as the contents of a truck arriving at a factory, a days worth of production, or the products stored in a warehouse. Ideally, the analyst would like to analyze every part of the material to obtain an accurate measure of the property of interest, but in most cases this is practically impossible. Many analytical techniques destroy the food and so there would be nothing left to sell if it were all analyzed. Another problem is that many analytical techniques are time consuming, expensive or labor intensive and so it is not economically feasible to analyze large amounts of material. It is therefore normal practice to select a fraction of the whole material for analysis, and to assume that its properties are representative of the whole material. Selection of an appropriate fraction of the whole material is one of the most important stages of food analysis procedures, and can lead to large errors when not carried out correctly.

Populations, Samples and Laboratory Samples. It is convenient to define some terms used to describe the characteristics of a material whose properties are going to be analyzed.

- *Population.* The whole of the material whose properties we are trying to obtain an estimate of is usually referred to as the *"population"*.
- *Sample.* Only a fraction of the population is usually selected for analysis, which is referred to as the *"sample"*. The sample may be comprised of one or more *sub-samples* selected from different regions within the population.

- *Laboratory Sample*. The sample may be too large to conveniently analyze using a laboratory procedure and so only a fraction of it is actually used in the final laboratory analysis. This fraction is usually referred to as the *"laboratory sample"*.

The primary objective of sample selection is to ensure that the properties of the *laboratory sample* are representative of the properties of the *population,* otherwise erroneous results will be obtained. Selection of a limited number of samples for analysis is of great benefit because it allows a reduction in time, expense and personnel required to carry out the analytical procedure, while still providing useful information about the properties of the population. Nevertheless, one must always be aware that analysis of a limited number of samples can only give an *estimate* of the true value of the whole population.

Sampling Plans. To ensure that the estimated value obtained from the *laboratory sample* is a good representation of the true value of the *population* it is necessary to develop a "*sampling plan"*. A sampling plan should be a clearly written document that contains precise details that an analyst uses to decide the *sample size*, the *locations* from which the sample should be selected, the *method* used to collect the sample, and the method used to preserve them prior to analysis. It should also stipulate the required documentation of procedures carried out during the sampling process. The choice of a particular sampling plan depends on the purpose of the analysis, the property to be measured, the nature of the total population and of the individual samples, and the type of analytical technique used to characterize the samples. For certain products and types of populations sampling plans have already been developed and documented by various organizations which authorize official methods, *e.g.*, the Association of Official Analytical Chemists (AOAC). Some of the most important considerations when developing or selecting an appropriate sampling plan are discussed below.

2.2.1 Purpose of Analysis

The first thing to decide when choosing a suitable sampling plan is the purpose of the analysis. Samples are analyzed for a number of different reasons in the food industry and this affects the type of sampling plan used:

- *Official samples.* Samples may be selected for official or legal requirements by government laboratories. These samples are analyzed to ensure that manufacturers are supplying safe foods that meet legal and labeling requirements. An officially

sanctioned sampling plan and analytical protocol is often required for this type of analysis.

- *Raw materials*. Raw materials are often analyzed before acceptance by a factory, or before use in a particular manufacturing process, to ensure that they are of an appropriate quality.
- *Process control samples*. A food is often analyzed during processing to ensure that the process is operating in an efficient manner. Thus if a problem develops during processing it can be quickly detected and the process adjusted so that the properties of the sample are not adversely effected. Techniques used to monitor process control must be capable of producing precise results in a short time. Manufacturers can either use analytical techniques that measure the properties of foods on-line, or they can select and remove samples and test them in a quality assurance laboratory.
- *Finished products.* Samples of the final product are usually selected and tested to ensure that the food is safe, meets legal and labeling requirements, and is of a high and consistent quality. Officially sanctioned methods are often used for determining nutritional labeling.
- *Research and Development.* Samples are analyzed by food scientists involved in fundamental research or in product development. In many situations it is not necessary to use a sampling plan in *R&D* because only small amounts of materials with well-defined properties are analyzed.

2.2.2 Nature of Measured Property

Once the reason for carrying out the analysis has been established it is necessary to clearly specify the particular property that is going to be measured, *e.g.,* color, weight, presence of extraneous matter, fat content or microbial count. The properties of foods can usually be classified as either *attributes* or *variables.* An attribute is something that a product either does or does not have, *e.g.,* it does or does not contain a piece of glass, or it is or is not spoilt. On the other hand, a *variable* is some property that can be measured on a continuous scale, such as the weight, fat content or moisture content of a material. Variable sampling usually requires less samples than attribute sampling.

The type of property measured also determines the seriousness of the outcome if the properties of the *laboratory sample* do not represent those of the *population*. For example, if the property measured is the presence of a harmful substance (such as bacteria, glass or toxic chemicals), then the

seriousness of the outcome if a mistake is made in the sampling is much greater than if the property measured is a quality parameter (such as color or texture). Consequently, the sampling plan has to be much more rigorous for detection of potentially harmful substances than for quantification of quality parameters.

2.2.3 Nature of Population

It is extremely important to clearly define the nature of the population from which samples are to be selected when deciding which type of sampling plan to use. Some of the important points to consider are listed below:

- A population may be either *finite* or *infinite*. A finite population is one that has a definite size, *e.g.*, a truckload of apples, a tanker full of milk, or a vat full of oil. An infinite population is one that has no definite size, *e.g.*, a conveyor belt that operates continuously, from which foods are selected periodically. Analysis of a finite population usually provides information about the properties of the population, whereas analysis of an infinite population usually provides information about the properties of the process. To facilitate the development of a sampling plan it is usually convenient to divide an "infinite" population into a number of finite populations, *e.g.*, all the products produced by one shift of workers, or all the samples produced in one day.
- A population may be either *continuous* or *compartmentalized.* A continuous population is one in which there is no physical separation between the different parts of the sample, *e.g.*, liquid milk or oil stored in a tanker. A compartmentalized population is one that is split into a number of separate sub-units, *e.g.*, boxes of potato chips in a truck, or bottles of tomato ketchup moving along a conveyor belt. The number and size of the individual sub-units determines the choice of a particular sampling plan.
- A population may be either *homogenous* or *heterogeneous.* A homogeneous population is one in which the properties of the individual samples are the same at every location within the material (*e.g.* a tanker of well stirred liquid oil), whereas a heterogeneous population is one in which the properties of the individual samples vary with location (*e.g.* a truck full of potatoes, some of which are bad). If the properties of a population were

homogeneous then there would be no problem in selecting a sampling plan because every individual sample would be representative of the whole population. In practice, most populations are heterogeneous and so we must carefully select a number of individual samples from different locations within the population to obtain an indication of the properties of the total population.

2.2.4 Nature of Test Procedure

The nature of the procedure used to analyze the food may also determine the choice of a particular sampling plan, *e.g.*, the speed, precision, accuracy and cost per analysis, or whether the technique is destructive or non-destructive. Obviously, it is more convenient to analyze the properties of many samples if the analytical technique used is capable of rapid, low cost, nondestructive and accurate measurements.

2.2.5. Developing a Sampling Plan

After considering the above factors one should be able to select or develop a sampling plan which is most suitable for a particular application. Different sampling plans have been designed to take into account differences in the types of samples and populations encountered, the information required and the analytical techniques used. Some of the features that are commonly specified in official sampling plans are listed below.

Sample size. The size of the sample selected for analysis largely depends on the expected variations in properties within a population, the seriousness of the outcome if a bad sample is not detected, the cost of analysis, and the type of analytical technique used. Given this information it is often possible to use statistical techniques to design a sampling plan that specifies the minimum number of sub-samples that need to be analyzed to obtain an accurate representation of the population. Often the size of the sample is impractically large, and so a process known as *sequential sampling* is used. Here sub-samples selected from the population are examined sequentially until the results are sufficiently definite from a statistical viewpoint. For example, sub-samples are analyzed until the ratio of good ones to bad ones falls within some statistically predefined value that enables one to confidently reject or accept the population.

Sample location. In homogeneous populations it does not matter where the sample is taken from because all the sub-samples have the same properties. In heterogeneous

populations the location from which the sub-samples are selected is extremely important. In *random sampling* the sub-samples are chosen randomly from any location within the material being tested. Random sampling is often preferred because it avoids human bias in selecting samples and because it facilitates the application of statistics. In s*ystematic sampling* the samples are drawn systematically with location or time, *e.g.*, every 10th box in a truck may be analyzed, or a sample may be chosen from a conveyor belt every 1 minute. This type of sampling is often easy to implement, but it is important to be sure that there is not a correlation between the sampling rate and the sub-sample properties. In *judgment sampling* the sub-samples are drawn from the whole population using the judgment and experience of the analyst. This could be the easiest sub-sample to get to, such as the boxes of product nearest the door of a truck. Alternatively, the person who selects the sub-samples may have some experience about where the worst sub-samples are usually found, *e.g.*, near the doors of a warehouse where the temperature control is not so good. It is not usually possible to apply proper statistical analysis to this type of sampling, since the sub-samples selected are not usually a good representation of the population.

Sample collection. Sample selection may either be carried out manually by a human being or by specialized mechanical sampling devices. Manual sampling may involve simply picking a sample from a conveyor belt or a truck, or using special cups or containers to collect samples from a tank or sack. The manner in which samples are selected is usually specified in sampling plans.

2.3 Preparation of Laboratory Samples

Once we have selected a sample that represents the properties of the whole population, we must prepare it for analysis in the laboratory. The preparation of a sample for analysis must be done very carefully in order to make accurate and precise measurements.

2.3.1 Making Samples Homogeneous

The food material within the *sample* selected from the *population* is usually heterogeneous, *i.e.*, its properties vary from one location to another. Sample heterogeneity may either be caused by variations in the properties of different units within the sample (*inter-unit* variation) and/or it may be caused by variations within the individual units in the sample (*intra-unit* variation). The units in the sample could be apples,

potatoes, bottles of ketchup, containers of milk etc. An example of inter-unit variation would be a box of oranges, some of good quality and some of bad quality. An example of intra-unit variation would be an individual orange, whose skin has different properties than its flesh. For this reason it is usually necessary to make samples *homogeneous* before they are analyzed, otherwise it would be difficult to select a representative *laboratory sample* from the *sample*. A number of mechanical devices have been developed for homogenizing foods, and the type used depends on the properties of the food being analyzed (*e.g.*, solid, semi-solid, liquid). Homogenization can be achieved using mechanical devices (*e.g.*, grinders, mixers, slicers, blenders), enzymatic methods (*e.g.*, proteases, cellulases, lipases) or chemical methods (*e.g.*, strong acids, strong bases, detergents).

2.3.2. Reducing Sample Size

Once the sample has been made homogeneous, a small more manageable portion is selected for analysis. This is usually referred to as a *laboratory sample,* and ideally it will have properties which are representative of the population from which it was originally selected. Sampling plans often define the method for reducing the size of a sample in order to obtain reliable and repeatable results.

2.3.3. Preventing Changes in Sample

Once we have selected our sample we have to ensure that it does not undergo any significant changes in its properties from the moment of sampling to the time when the actual analysis is carried out, *e.g.*, enzymatic, chemical, microbial or physical changes. There are a number of ways these changes can be prevented.

- *Enzymatic Inactivation.* Many foods contain active enzymes they can cause changes in the properties of the food prior to analysis, *e.g.*, proteases, cellulases, lipases, etc. If the action of one of these enzymes alters the characteristics of the compound being analyzed then it will lead to erroneous data and it should therefore be inactivated or eliminated. Freezing, drying, heat treatment and chemical preservatives (or a combination) are often used to control enzyme activity, with the method used depending on the type of food being analyzed and the purpose of the analysis.
- *Lipid Protection.* Unsaturated lipids may be altered by various oxidation reactions. Exposure to light, elevated temperatures, oxygen or pro-oxidants can

increase the rate at which these reactions proceed. Consequently, it is usually necessary to store samples that have high unsaturated lipid contents under nitrogen or some other inert gas, in dark rooms or covered bottles and in refrigerated temperatures. Providing that they do not interfere with the analysis antioxidants may be added to retard oxidation.

- *Microbial Growth and Contamination.* Microorganisms are present naturally in many foods and if they are not controlled they can alter the composition of the sample to be analyzed. Freezing, drying, heat treatment and chemical preservatives (or a combination) are often used to control the growth of microbes in foods.
- *Physical Changes.* A number of physical changes may occur in a sample, *e.g.*, water may be lost due to evaporation or gained due to condensation; fat or ice may melt or crystallize; structural properties may be disturbed. Physical changes can be minimized by controlling the temperature of the sample, and the forces that it experiences.

2.3.4. Sample Identification

Laboratory samples should always be labeled carefully so that if any problem develops its origin can easily be identified. The information used to identify a sample includes: a) Sample description, b) Time sample was taken, c) Location sample was taken from, d) Person who took the sample, and, e) Method used to select the sample. The analyst should always keep a detailed notebook clearly documenting the sample selection and preparation procedures performed and recording the results of any analytical procedures carried out on each sample. Each sample should be marked with a *code* on its label that can be correlated to the notebook. Thus if any problem arises, it can easily be identified.

2.4. Data Analysis and Reporting

Food analysis usually involves making a number of repeated measurements on the same sample to provide confidence that the analysis was carried out correctly and to obtain a best estimate of the value being measured and a statistical indication of the reliability of the value. A variety of statistical techniques are available that enable us to obtain this information about the laboratory sample from multiple measurements.

2.4.1. Measure of Central Tendency of Data

The most commonly used parameter for representing the overall properties of a number of measurements is the *mean:*

$$\bar{x} = \frac{x_1 + x_2 + x_3 + \ldots x_n}{n} = \frac{\sum_{i=1}^{n} x_i}{n} \qquad (1)$$

Here n is the total number of measurements, x_i is the individually measured values and $\bar{x}$ is the mean value.

The mean is the *best experimental estimate* of the value that can be obtained from the measurements. It does not necessarily have to correspond to the *true* value of the parameter one is trying to measure. There may be some form of systematic error in our analytical method that means that the measured value is not the same as the true value (see below). *Accuracy* refers to how closely the *measured* value agrees with the *true* value. The problem with determining the accuracy is that the true value of the parameter being measured is often not known. Nevertheless, it is sometimes possible to purchase or prepare standards that have known properties and analyze these standards using the same analytical technique as used for the unknown food samples. The absolute error E_{abs}, which is the difference between the true value (x_{true}) and the measured value (x_i), can then be determined: $E_{abs} = (x_i - x_{true})$. For these reasons, analytical instruments should be carefully maintained and frequently calibrated to ensure that they are operating correctly.

2.4.2. Measure of Spread of Data

The *spread of the data is* a measurement of how closely together repeated measurements are to each other. The *standard deviation* is the most commonly used measure of the spread of experimental measurements. This is determined by assuming that the experimental measurements vary randomly about the mean, so that they can be represented by a normal distribution. The standard deviation *SD* of a set of experimental measurements is given by the following equation:

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \qquad (2)$$

Measured values within the specified range:

$\bar{x} \pm SD$ means 68% values within range (x - SD) to (x + SD)

$\bar{x} \pm 2SD$ means 95% values within range (x - 2SD) to (x + 2SD)

$\bar{x} \pm 3SD$ means >99% values within range (x - 3SD) to (x + 3SD)

Another parameter that is commonly used to provide an indication of the relative spread of the data around the mean is the *coefficient of variation, CV* = $[SD / \bar{x}] \times 100\%$.

2.4.3. Sources of Error

There are three common sources of error in any analytical technique:

- *Personal Errors (Blunders).* These occur when the analytical test is not carried out correctly: the wrong chemical reagent or equipment might have been used; some of the sample may have been spilt; a volume or mass may have been recorded incorrectly; etc. It is partly for this reason that analytical measurements should be repeated a number of times using freshly prepared laboratory samples. Blunders are usually easy to identify and can be eliminated by carrying out the analytical method again more carefully.
- *Random Errors.* These produce data that vary in a non-reproducible fashion from one measurement to the next *e.g.*, instrumental noise. This type of error determines the standard deviation of a measurement. There may be a number of different sources of random error and these are accumulative (see “Propagation of Errors”).
- *Systematic Errors.* A systematic error produces results that consistently deviate from the true answer in some systematic way, *e.g.*, measurements may always be 10% too high. This type of error would occur if the volume of a pipette was different from the stipulated value. For example, a nominally 100 cm^3 pipette may always deliver 101 cm^3 instead of the correct value.

To make accurate and precise measurements it is important when designing and setting up an analytical procedure to identify the various sources of error and to minimize their effects. Often, one particular step will be the largest source of error, and the best improvement in accuracy or precision can be achieved by minimizing the error in this step.

2.4.4. Propagation of Errors

Most analytical procedures involve a number of steps (*e.g.*, weighing, volume measurement, reading dials), and there will be an error associated with each step. These individual errors accumulate to determine the overall error in the final result. For random errors there are a number of simple rules that can be followed to calculate the error in the final result:

Addition (Z = X+Y) and Subtraction (Z = X-Y):

$$\Delta Z = \sqrt{\Delta X^2 + \Delta Y^2} \qquad (3)$$

Multiplication (Z = XY) and Division (Z = X/Y):

$$\frac{\Delta Z}{Z} = \sqrt{\left(\frac{\Delta X}{X}\right)^2 + \left(\frac{\Delta Y}{Y}\right)^2} \qquad (4)$$

Here, ΔX is the standard deviation of the mean value X, ΔY is the standard deviation of the mean value Y, and ΔZ is the standard deviation of the mean value Z. These simple rules should be learnt and used when calculating the overall error in a final result.

As an example, let us assume that we want to determine the **fat content** of a food and that we have previously measured the mass of extracted fat extracted from the food (M_E) and the initial mass of the food (M_I):

$M_E = 3.1 \pm 0.3$ g

$M_I = 10.5 \pm 0.7$ g

% Fat Content = $100 \times M_E / M_I$

To calculate the mean and standard deviation of the fat content we need to use the multiplication rule ($Z=X/Y$) given by Equation 4. Initially, we assign values to the various parameters in the appropriate propagation of error equation:

$X = 3.1$; $\Delta X = 0.3$

$Y = 10.5$; $\Delta Y = 0.7$

% Fat Content = $Z = 100 \times X/Y = 100 \times 3.1/10.5 = 29.5\%$

$\Delta Z = Z \sqrt{[(\Delta X/X)^2+(\Delta Y/Y)^2]} = 29.5\% \sqrt{[(0.3/3.1)^2+(0.7/10.5)^2]} = 3.5\%$

Hence, the fat content of the food is $29.5 \pm 3.5\%$. In reality, it may be necessary to carry out a number of different steps in a calculation, some that involve addition/subtraction and some that involve multiplication/division. When carrying out multiplication/division calculations it is necessary to ensure that all appropriate addition/subtraction calculations have been completed first.

2.4.5. Significant Figures and Rounding

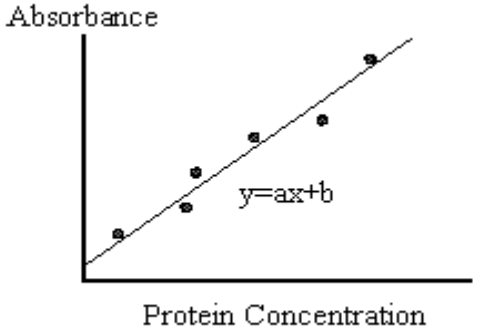
The number of *significant figures* used in reporting a final result is determined by the standard deviation of the measurements. A final result is reported to the correct number of significant figures when it contains all the digits that are known to be correct, plus a final one that is known to be uncertain. For example, a reported value of 12.13, means that the 12.1 is known to be correct but the 3 at the end is uncertain, it could be either a 2 or a 4 instead.

For multiplication ($Z = X \times Y$) and division ($Z = X/Y$), the significant figures in the final result (Z) should be equal to the significant figures in the number from which it was calculated (X or Y) that has the lowest significant figures. For example, 12.312 (5 significant figures) x 31.1 (3 significant figures) = 383 (3 significant figures). For addition ($Z = X + Y$) and subtraction ($Z = X - Y$), the significant figures in the final result (Z) are determined by the number from which it was calculated (X or Y) that has the last significant figure in the highest decimal column. For example, 123.4567 (last significant figure in the "0.0001" decimal column) + 0.31 (last significant figure in the "0.01" decimal column) = 123.77 (last significant figure in the "0.01" decimal column). Or, 1310 (last significant figure in the "10" decimal column) + 12.1 (last significant figure in the "0.1" decimal column) = 1320 (last significant figure in the "10" decimal column).

When rounding numbers: always round any number with a final digit less than 5 downwards, and 5 or more upwards, *e.g.* 23.453 becomes 23.45; 23.455 becomes 23.46; 23.458 becomes 23.46. It is usually desirable to carry extra digits throughout the calculations and then round off the final result.

2.4.6. Standard Curves: Regression Analysis

When carrying out certain analytical procedures it is necessary to prepare standard curves that are used to determine some property of an unknown material. A series of calibration experiments is carried out using samples with known properties and a standard curve is plotted from this data. For example, a series of protein solutions with known concentration of protein could be prepared and their absorbance of electromagnetic radiation at 280 nm could be measured using a UV-visible spectrophotometer. For dilute protein solutions there is a linear relationship between absorbance and protein concentration:



A best-fit line is drawn through the date using *regression analysis*, which has a gradient of *a* and a y-intercept of *b*. The concentration of protein in an unknown sample can then be determined by measuring its absorbance: $x = (y-b)/a$, where in this example *x* is the protein concentration and *y* is the absorbance. How well the straight-line fits the experimental data is expressed by the correlation coefficient r^2, which has a value between 0 and 1. The closer the value is to 1 the better the fit between the straight line and the experimental values: $r^2 = 1$ is a perfect fit. Most modern calculators and spreadsheet programs have routines that can be used to automatically determine the regression coefficient, the slope and the intercept of a set of data.

2.4.7. Rejecting Data

When carrying out an experimental analytical procedure it will sometimes be observed that one of the measured values is very different from all of the other values, *e.g.*, as the result of a "blunder" in the analytical procedure. Occasionally, this value may be treated as being incorrect, and it can be rejected. There are certain rules based on statistics that allow us to decide whether a particular point can be rejected or not. A test called the *Q-test* is commonly used to decide whether an experimental value can be rejected or not.

$$Q = \frac{X_{BAD} - X_{NEXT}}{X_{HIGH} - X_{LOW}}$$

Here X_{BAD} is the questionable value, X_{NEXT} is the next closet value to X_{BAD}, X_{HIGH} is the highest value of the data set and X_{LOW} is the lowest value of the data set. If the *Q-value* is higher than the value given in a *Q-test table* for the number of samples being analyzed then it can be rejected:

Number of Observations	Q-value for Data Rejection (90% confidence level)
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

For example, if five measurements were carried out and one measurement was very different from the rest (*e.g.*, 20,22,25,50,21), having a Q-value of 0.84, then it could be safely rejected (because it is higher than the value of 0.64 given in the Q-test table for five observations).

References

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3. Determination of Moisture and Total Solids

3.1 Introduction

Moisture content is one of the most commonly measured properties of food materials. It is important to food scientists for a number of different reasons:

- *Legal and Labeling Requirements.* There are legal limits to the maximum or minimum amount of water that must be present in certain types of food.
- *Economic.* The cost of many foods depends on the amount of water they contain - water is an inexpensive ingredient, and manufacturers often try to incorporate as much as possible in a food, without exceeding some maximum legal requirement.
- *Microbial Stability.* The propensity of microorganisms to grow in foods depends on their water content. For this reason many foods are dried below some critical moisture content.
- *Food Quality.* The texture, taste, appearance and stability of foods depends on the amount of water they contain.
- *Food Processing Operations.* A knowledge of the moisture content is often necessary to predict the behavior of foods during processing, *e.g.* mixing, drying, flow through a pipe or packaging.

It is therefore important for food scientists to be able to reliably measure moisture contents. A number of analytical techniques have been developed for this purpose, which vary in their accuracy, cost, speed, sensitivity, specificity, ease of operation, *etc*. The choice of an analytical procedure for a particular application depends on the nature of the food being analyzed and the reason the information is needed.

3.2 Properties of Water in Foods

The moisture content of a food material is defined through the following equation:

$$\%\text{Moisture} = (m_w/m_{sample}) \times 100$$

Where m_w is the mass of the water and m_{sample} is the mass of the sample. The mass of water is related to the number of water molecules (n_W) by the following expression: $m_w = n_w M_w / N_A$, where M_w is the molecular weight of water (18.0 g per mole) and N_A is Avadagro's number (6.02×10^{23} molecules per mole). In principle, the moisture

content of a food can therefore be determined accurately by measuring the number or mass of water molecules present in a known mass of sample. It is not possible to directly measure the number of water molecules present in a sample because of the huge number of molecules involved. A number of analytical techniques commonly used to determine the moisture content of foods are based on determinations of the mass of water present in a known mass of sample. Nevertheless, as we will see later, there are a number of practical problems associated with these techniques that make highly accurate determinations of moisture content difficult or that limit their use for certain applications. For these reasons, a number of other analytical methods have been developed to measure the moisture content of foods that do not rely on direct measurement of the mass of water in a food. Instead, these techniques are based on the fact that the water in a food can be distinguished from the other components in some measurable way.

An appreciation of the principles, advantages and limitations of the various analytical techniques developed to determine the moisture content of foods depends on an understanding of the molecular characteristics of water. A water molecule consists of an oxygen atom covalently bound to two hydrogen atoms (H_2O). Each of the hydrogen atoms has a small positive charge ($\delta+$), while the oxygen atom has two lone pairs of electrons that each has a small negative charge ($\delta-$). Consequently, water molecules are capable of forming relatively strong hydrogen bonds ($O\text{-}H^{\delta+} \leftrightarrow {}^{\delta-}O$) with four neighboring water molecules. The strength and directionality of these hydrogen bonds are the origin of many of the unique physicochemical properties of water. The development of analytical techniques to determine the moisture content of foods depends on being able to distinguish water (the "analyte") from the other components in the food (the "matrix"). The characteristics of water that are most commonly used to achieve this are: its relatively low boiling point; its high polarity; its ability to undergo unique chemical reactions with certain reagents; its unique electromagnetic absorption spectra; and, its characteristic physical properties (density, compressibility, electrical conductivity and refractive index).

Despite having the same chemical formula (H_2O) the water molecules in a food may be present in a variety of different molecular environments depending on their interaction with the surrounding molecules. The water molecules in these different environments normally have different physiochemical properties:

- *Bulk water*. Bulk water is free from any other constituents, so that each water molecule is surrounded only by other water molecules. It therefore has physicochemical properties that are the same as those of pure water, *e.g.*, melting point, boiling point, density, compressibility, heat of vaporization, electromagnetic absorption spectra.
- *Capillary or trapped water.* Capillary water is held in narrow channels between certain food components because of capillary forces. Trapped water is held within spaces within a food that are surrounded by a physical barrier that prevents the water molecules from easily escaping, *e.g.*, an emulsion droplet or a biological cell. The majority of this type of water is involved in normal water-water bonding and so it has physicochemical properties similar to that of bulk water.
- *Physically bound water.* A significant fraction of the water molecules in many foods are not completely surrounded by other water molecules, but are in

molecular contact with other food constituents, *e.g.* proteins, carbohydrates or minerals. The bonds between water molecules and these constituents are often significantly different from normal water-water bonds and so this type of water has different physicochemical properties than bulk water *e.g.*, melting point, boiling point, density, compressibility, heat of vaporization, electromagnetic absorption spectra.

- *Chemically bound water.* Some of the water molecules present in a food may be chemically bonded to other molecules as water of crystallization or as hydrates, *e.g.* $NaSO_4.10H_20$. These bonds are much stronger than the normal water-water bond and therefore chemically bound water has very different physicochemical properties to bulk water, *e.g.*, lower melting point, higher boiling point, higher density, lower compressibility, higher heat of vaporization, different electromagnetic absorption spectra.

Foods are heterogeneous materials that contain different proportions of chemically bound, physically bound, capillary, trapped or bulk water. In addition, foods may contain water that is present in different physical states: gas, liquid or solid. The fact that water molecules can exist in a number of different molecular environments, with different physicochemical properties, can be problematic for the food analyst trying to accurately determine the moisture content of foods. Many analytical procedures developed to measure moisture content are more sensitive to water in certain types of molecular environment than to water in other types of molecular environment. This means that the measured value of the moisture content of a particular food may depend on the experimental technique used to carry out the measurement. Sometimes food analysts are interested in determining the amounts of water in specific molecular environments (*e.g.*, physically bound water), rather than the total water content. For example, the rate of microbial growth in a food depends on the amount of bulk water present in a food, and not necessarily on the total amount of water present. There are analytical techniques available that can provide some information about the relative fractions of water in different molecular environments (*e.g.*, DSC, NMR, vapor pressure).

3.3. Sample preparation

Selection of a representative sample, and prevention of changes in the properties of the sample prior to analysis, are two major potential sources of error in any food analysis procedure. When determining the moisture content of a food it is important to prevent any loss or gain of water. For this reason, exposure of a sample to the atmosphere, and excessive temperature fluctuations, should be minimized. When samples are stored in containers it is common practice to fill the container to the top to prevent a large headspace, because this reduces changes in the sample due to equilibration with its environment. The most important techniques developed to measure the moisture content of foods are discussed below.

3.4. Evaporation methods

3.4.1. Principles

These methods rely on measuring the mass of water in a known mass of sample. The moisture content is determined by measuring the mass of a food before and after the water is removed by evaporation:

$$\%\text{Moisture} = \frac{M_{\text{INITIAL}} - M_{\text{DRIED}}}{M_{\text{INITIAL}}} \times 100$$

Here, M_{INITIAL} and M_{DRIED} are the mass of the sample before and after drying, respectively. The basic principle of this technique is that water has a lower boiling point than the other major components within foods, *e.g.*, lipids, proteins, carbohydrates and minerals. Sometimes a related parameter, known as the *total solids*, is reported as a measure of the moisture content. The total solids content is a measure of the amount of material remaining after all the water has been evaporated:

$$\%\text{Total Solids} = \frac{M_{\text{DRIED}}}{M_{\text{INITIAL}}} \times 100$$

Thus, %Total solids = (100 - %Moisture). To obtain an accurate measurement of the moisture content or total solids of a food using evaporation methods it is necessary to remove all of the water molecules that were originally present in the food, without changing the mass of the food matrix. This is often extremely difficult to achieve in practice because the high temperatures or long times required to remove all of the water molecules would lead to changes in the mass of the food matrix, *e.g.*, due to volatilization or chemical changes of some components. For this reason, the drying conditions used in evaporation methods are usually standardized in terms of temperature and time so as to obtain results that are as accurate and reproducible as possible given the practical constraints. Using a standard method of sample preparation and analysis helps to minimize sample-to-sample variations within and between laboratories.

3.4.2. Evaporation Devices

The thermal energy used to evaporate the water from a food sample can be provided directly (*e.g.*, transfer of heat from an oven to a food) or indirectly (*e.g.*, conversion of electromagnetic radiation incident upon a food into heat due to absorption of energy by the water molecules).

Convection and forced draft ovens. Weighed samples are placed in an oven for a specified time and temperature (*e.g.* 3 hours at 100 ^{o}C) and their dried mass is determined, or they are dried until they reach constant mass. The thermal energy used to evaporate the water is applied directly to the sample *via* the shelf and air that surround it. There are often considerable temperature variations within convection ovens, and so precise measurements are carried out using *forced draft ovens* that circulate the air so as to achieve a more uniform temperature distribution within the oven. Samples that contain significant quantities of carbohydrates that might undergo chemical changes or volatile materials other than water should not be dried in a convection or forced draft oven. Many official methods of analysis are based on forced draft ovens.

Vacuum oven. Weighed samples are placed under reduced pressure (typically 25-100 mm Hg) in a vacuum oven for a specified time and temperature and their dried mass is determined. The thermal energy used to evaporate the water is applied directly to the sample *via* the metallic shelf that it sits upon. There is an air inlet and outlet to carry the moisture lost from the sample out of the vacuum oven, which prevents the accumulation of moisture within the oven. The boiling point of water is reduced when it is placed under vacuum. Drying foods in a vacuum oven therefore has a number of advantages over conventional oven drying techniques. If the sample is heated at the same temperature, drying can be carried out much quicker. Alternatively, lower temperatures can be used to remove the moisture (*e.g.* 70^{o}C instead of 100 ^{o}C), and so problems associated with degradation of heat labile substances can be reduced. A number of vacuum oven methods are officially recognized.

Microwave oven. Weighed samples are placed in a microwave oven for a specified time and power-level and their dried mass is weighed. Alternatively, weighed samples may be dried until they reach a constant final mass - analytical microwave ovens containing balances to continuously monitor the weight of a food during drying are commercially available. The water molecules in the food evaporate because they absorb microwave energy, which causes them to become thermally excited. The major advantage of microwave methods over other drying methods is that they are simple to use and rapid to carry out. Nevertheless, care must be taken to standardize the drying procedure and ensure that the microwave energy is applied evenly across the sample. A number of microwave oven drying methods are officially recognized.

Infrared lamp drying. The sample to be analyzed is placed under an infrared lamp and its mass is recorded as a function of time. The water molecules in the food evaporate because they absorb infrared energy, which causes them to become thermally excited. One of the major advantages of infrared drying methods is that moisture contents can be determined rapidly using inexpensive equipment, *e.g.,* 10-25 minutes. This is because the IR energy penetrates into the sample, rather than having to be conducted and convected inwards from the surface of the sample. To obtain reproducible measurements it is important to control the distance between the sample and the IR lamp and the dimensions of the sample. IR drying methods are not officially recognized for moisture content determinations because it is difficult to standardize the procedure. Even so, it is widely used in industry because of its speed and ease of use.

3.4.3. Practical Considerations

1. *Sample dimensions.* The rate and extent of moisture removal depends on the size and shape of the sample, and how finely it is ground. The greater the surface area of material exposed to the environment, the faster the rate of moisture removal.
2. *Clumping and surface crust formation*. Some samples tend to clump together or form a semi-permeable surface crust during the drying procedure. This can lead to erroneous and irreproducible results because the loss of moisture is restricted by the clumps or crust. For this reason samples are often mixed with dried sand to prevent clumping and surface crust formation.

3. *Elevation of boiling point.* Under normal laboratory conditions pure water boils at 100 ^{o}C. Nevertheless, if solutes are present in a sample the boiling point of water is elevated. This is because the partial vapor pressure of water is decreased and therefore a higher temperature has to be reached before the vapor pressure of the system equals the atmospheric pressure. Consequently, the rate of moisture loss from the sample is slower than expected. The boiling point of water containing solutes (T_b) is given by the expression, $T_b = T_0 + 0.51m$, where T_0 is the boiling point of pure water and m is the molality of solute in solution (mol/kg of solvent).
4. *Water type.* The ease at which water is removed from a food by evaporation depends on its interaction with the other components present. Free water is most easily removed from foods by evaporation, whereas more severe conditions are needed to remove chemically or physically bound water. Nevertheless, these more extreme conditions can cause problems due to degradation of other ingredients which interfere with the analysis (see below).
5. *Decomposition of other food components.* If the temperature of drying is too high, or the drying is carried out for too long, there may be decomposition of some of the heat-sensitive components in the food. This will cause a change in the mass of the food matrix and lead to errors in the moisture content determination. It is therefore normally necessary to use a compromise time and temperature, which are sufficient to remove most of the moisture, but not too long to cause significant thermal decomposition of the food matrix. One example of decomposition that interferes with moisture content determinations is that of carbohydrates.

   $$C_6H_{12}O_6 \xrightarrow{\text{heat}} 6C + 6\ H_2O$$

   The water that is released by this reaction is not the water we are trying to measure and would lead to an overestimation of the true moisture content. On the other hand, a number of chemical reactions that occur at elevated temperatures lead to water absorption, *e.g.*, sucrose hydrolysis (sucrose + H_2O $\xrightarrow{\text{heat}}$ fructose + glucose), and therefore lead to an underestimation of the true moisture content. Foods that are particularly susceptible to thermal decomposition should be analyzed using alternative methods, *e.g.* chemical or physical.

6. *Volatilization of other food components.* It is often assumed that the weight loss of a food upon heating is entirely due to evaporation of the water. In practice, foods often contain other volatile constituents that can also be lost during heating, *e.g.*, flavors or odors. For most foods, these volatiles only make up a very small proportion and can therefore be ignored. For foods that do contain significant amounts of volatile components (*e.g.* spices and herbs) it is necessary to use alternative methods to determine their moisture content, *e.g.*, distillation, chemical or physical methods.
7. *High moisture samples.* Food samples that have high moisture contents are usually dried in two stages to prevent "spattering" of the sample, and accumulation of moisture in the oven. Spattering is the process whereby some of the water jumps out of the food sample during drying, carrying other food

constituents with it. For example, most of the moisture in milk is removed by heating on a steam bath prior to completing the drying in an oven.

8. *Temperature and power level variations.* Most evaporation methods stipulate a definite temperature or power level to dry the sample so as to standardize the procedure and obtain reproducible results. In practice, there are often significant variations in temperatures or power levels within an evaporation instrument, and so the efficiency of the drying procedure depends on the precise location of the sample within the instrument. It is therefore important to carefully design and operate analytical instruments so as to minimize these temperature or power level variations.
9. *Sample pans.* It is important to use appropriate pans to contain samples, and to handle them correctly, when carrying out a moisture content analysis. Typically aluminum pans are used because they are relatively cheap and have a high thermal conductivity. These pans usually have lids to prevent spattering of the sample, which would lead to weight loss and therefore erroneous results. Pans should be handled with tongs because fingerprints can contribute to the mass of a sample. Pans should be dried in an oven and stored in a descicator prior to use to ensure that no residual moisture is attached to them.

3.4.4. Advantages and Disadvantages

- - *Advantages:* Precise; Relatively cheap; Easy to use; Officially sanctioned for many applications; Many samples can be analyzed simultaneously
- - *Disadvantages:* Destructive; Unsuitable for some types of food; Time consuming

3.5. Distillation Methods

3.5.1. Principles

Distillation methods are based on *direct* measurement of the amount of water removed from a food sample by evaporation: %Moisture = 100 ($M_{WATER}/M_{INITIAL}$). In contrast, evaporation methods are based on *indirect* measurement of the amount of water removed from a food sample by evaporation: %Moisture = 100 ($M_{INITIAL}$ - M_{DRIED})/$M_{INITIAL}$. Basically, distillation methods involve heating a weighed food sample ($M_{INITIAL}$) in the presence of an organic solvent that is immiscible with water. The water in the sample evaporates and is collected in a graduated glass tube where its mass is determined (M_{WATER}).

3.5.2. Dean and Stark Method

Distillation methods are best illustrated by examining a specific example: the Dean and Stark method. A known weight of food is placed in a flask with an organic solvent such as xylene or toluene. The organic solvent must be insoluble with water; have a higher boiling point than water; be less dense than water; and be safe to use. The flask containing the sample and the organic solvent is attached to a condenser by

a side arm and the mixture is heated. The water in the sample evaporates and moves up into the condenser where it is cooled and converted back into liquid water, which then trickles into the graduated tube. When no more water is collected in the graduated tube, distillation is stopped and the volume of water is read from the tube.

3.5.3. Practical Considerations

There are a number of practical factors that can lead to erroneous results: (i) emulsions can sometimes form between the water and the solvent which are difficult to separate; (ii) water droplets can adhere to the inside of the glassware, (iii) decomposition of thermally labile samples can occur at the elevated temperatures used.

3.5.4. Advantages and Disadvantages

- - *Advantages:* Suitable for application to foods with low moisture contents; Suitable for application to foods containing volatile oils, such as herbs or spices, since the oils remain dissolved in the organic solvent, and therefore do not interfere with the measurement of the water; Equipment is relatively cheap, easy to setup and operate; Distillation methods have been officially sanctioned for a number of food applications.
- - *Disadvantages:* Destructive; Relatively time-consuming; Involves the use of flammable solvents; Not applicable to some types of foods.

3.6. Chemical Reaction Methods

Reactions between water and certain chemical reagents can be used as a basis for determining the concentration of moisture in foods. In these methods a chemical reagent is added to the food that reacts specifically with water to produce a measurable change in the properties of the system, *e.g.*, mass, volume, pressure, pH, color, conductivity. Measurable changes in the system are correlated to the moisture content using calibration curves. To make accurate measurements it is important that the chemical reagent reacts with all of the water molecules present, but not with any of the other components in the food matrix. Two methods that are commonly used in the food industry are the *Karl-Fisher titration* and *gas production* methods. Chemical reaction methods do not usually involve the application of heat and so they are suitable for foods that contain thermally labile substances that would change the mass of the food matrix on heating (*e.g.*, food containing high sugar concentrations) or foods that contain volatile components that might be lost by heating (*e.g.* spices and herbs).

3.6.1. Karl-Fisher method

The Karl-Fisher titration is often used for determining the moisture content of foods that have low water contents (*e.g.* dried fruits and vegetables, confectionary, coffee, oils and fats). It is based on the following reaction:

$$2H_2O + SO_2 + I_2 \rightarrow H_2SO_4 + 2HI$$

This reaction was originally used because HI is colorless, whereas I_2 is a dark reddish brown color, hence there is a measurable change in color when water reacts with the added chemical reagents. Sulfur dioxide and iodine are gaseous and would normally be lost from solution. For this reason, the above reaction has been modified by adding solvents (*e.g.*, C_5H_5N) that keep the S_2O and I_2 in solution, although the basic principles of the method are the same. The food to be analyzed is placed in a beaker containing solvent and is then titrated with *Karl Fisher reagent* (a solution that contains iodine). While any water remains in the sample the iodine reacts with it and the solution remains colorless (HI), but once all the water has been used up any additional iodine is observed as a dark red brown color (I_2). The volume of iodine solution required to titrate the water is measured and can be related to the moisture content using a pre-prepared calibration curve. The precision of the technique can be improved by using electrical methods to follow the end-point of the reaction, rather than observing a color change. Relatively inexpensive commercial instruments have been developed which are based on the Karl-Fisher titration, and some of these are fully automated to make them less labor intensive.

3.6.2. Gas production methods

Commercial instruments are also available that utilize specific reactions between chemical reagents and water that lead to the production of a gas. For example, when a food sample is mixed with powdered calcium carbide the amount of acetylene gas produced is related to the moisture content.

$$CaC_2 + 2H_2O \rightarrow C_2H_2(gas) + Ca(OH)_2$$

The amount of gas produced can be measured in a number of different ways, including (i) the volume of gas produced, (ii) the decrease in the mass of the sample after the gas is released, and (iii) the increase in pressure of a closed vessel containing the reactants.

3.7 Physical Methods

A number of analytical methods have been developed to determine the moisture content of foods that are based on the fact that water has appreciably different bulk physical characteristics than the food matrix, *e.g.* density, electrical conductivity or refractive index. These methods are usually only suitable for analysis of foods in which the composition of the food matrix does not change significantly, but the ratio of water-to-food matrix changes. For example, the water content of oil-in-water emulsions can be determined by measuring their density or electrical conductivity because the density and electrical conductivity of water are significantly higher than those of oil. If the composition of the food matrix changes as well as the water

content, then it may not be possible to accurately determine the moisture content of the food because more than one food composition may give the same value for the physical property being measured. In these cases, it may be possible to use a combination of two or more physical methods to determine the composition of the food, *e.g.*, density measurements in combination with electrical conductivity measurements.

3.8 Spectroscopic Methods

Spectroscopic methods utilize the interaction of electromagnetic radiation with materials to obtain information about their composition, *e.g.*, X-rays, UV-visible, NMR, microwaves and IR. The spectroscopic methods developed to measure the moisture content of foods are based on the fact that water absorbs electromagnetic radiation at characteristic wavelengths that are different from the other components in the food matrix. The most widely used physical methods are based on measurements of the absorption of microwave or infrared energy by foods. Microwave and infrared radiation are absorbed by materials due to their ability to promote the vibration and/or rotation of molecules. The analysis is carried out at a wavelength where the water molecules absorb radiation, but none of the other components in the food matrix do. A measurement of the absorption of radiation at this wavelength can then be used to determine the moisture content: the higher the moisture content, the greater the absorption. Instruments based on this principle are commercially available and can be used to determine the moisture content in a few minutes or less. It is important not to confuse infrared and microwave *absorption* methods with infrared lamp and microwave *evaporation* methods. The former use low energy waves that cause no physical or chemical changes in the food, whereas the latter use high-energy waves to evaporate the water. The major advantage of these methods is that they are capable of rapidly determining the moisture content of a food with little or no sample preparation and are therefore particularly useful for quality control purposes or rapid measurements of many samples.

3.9 Methods to Determine Water in Different Molecular Environments

The overall water content of a food is sometimes not a very reliable indication of the quality of a food because the water molecules may exist in different environments within foods, *e.g.*, "bound" or "free". Here "bound water" refers to water that is physically or chemically bound to other food components, whereas "free water" refers to bulk, capillary or entrapped water. For example, the microbial stability or physicochemical properties of a food are often determined by the amount of free water present, rather than by the total amount of water present. For this reason, it is often useful for food scientists to be able to determine the amount of water in different molecular environments within a food. A variety of analytical methods are available that can provide this type of information.

3.9.1. Vapor pressure methods

A physical parameter that is closely related to the amount of free water present in a food is the water activity:

$$a_w = \frac{P}{P_0}$$

where, P is the partial pressure of the water above the food and P_0 is the vapor pressure of pure water at the same temperature. Bound water is much less volatile than free water, and therefore the water activity gives a good indication of the amount of free water present. A variety of methods are available for measuring the water activity of a sample based on its vapor pressure. Usually, the sample to be analyzed is placed in a closed container and allowed to come into equilibrium with its environment. The water content in the headspace above the sample is then measured and compared to that of pure water under the same conditions.

3.9.2. Thermogravimetric methods

Thermogravimetric techniques can be used to continuously measure the mass of a sample as it is heated at a controlled rate. The temperature at which water evaporates depends on its molecular environment: free water normally evaporates at a lower temperature than bound water. Thus by measuring the change in the mass of a sample as it loses water during heating it is often possible to obtain an indication of the amounts of water present in different molecular environments.

3.9.3. Calorimetric methods

Calorimetric techniques such as differential scanning calorimetry (DSC) and differential thermal analysis (DTA) can be used to measure changes in the heat absorbed or released by a material as its temperature is varied at a controlled rate. The melting point of water depends on its molecular environment: free water normally melts at a higher temperature than bound water. Thus by measuring the enthalpy change of a sample with temperature it is possible to obtain an indication of the amounts of water present in different molecular environments.

Spectroscopic methods

The electromagnetic spectrum of water molecules often depends on their molecular environment, and so some spectroscopy techniques can be used to measure the amounts of water in different environments. One of the most widely used of these techniques is nuclear magnetic resonance (NMR). NMR can distinguish molecules within materials based on their molecular mobility, *i.e.*, the distance they move in a

given time. The molecular mobility of free water is appreciably higher than that of bound water and so NMR can be used to provide an indication of the concentrations of water in "free" and "bound" states.

4. Analysis of Ash and Minerals

4.1 Introduction

The "*ash content*" is a measure of the total amount of minerals present within a food, whereas the "*mineral content*" is a measure of the amount of specific inorganic components present within a food, such as Ca, Na, K and Cl. Determination of the ash and mineral content of foods is important for a number of reasons:

- *Nutritional labeling.* The concentration and type of minerals present must often be stipulated on the label of a food.
- *Quality.* The quality of many foods depends on the concentration and type of minerals they contain, including their taste, appearance, texture and stability.
- *Microbiological stability.* High mineral contents are sometimes used to retard the growth of certain microorganisms.
- *Nutrition.* Some minerals are essential to a healthy diet (*e.g.*, calcium, phosphorous, potassium and sodium) whereas others can be toxic (*e.g.*, lead, mercury, cadmium and aluminum).
- *Processing.* It is often important to know the mineral content of foods during processing because this affects the physicochemical properties of foods.

4.2. Determination of Ash Content

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. Analytical techniques for providing information about the total mineral content are based on the fact that the minerals (the "analyte") can be distinguished from all the other components (the "matrix") within a food in some measurable way. The most widely used methods are based on the fact that minerals are not destroyed by heating, and that they have a low volatility compared to other food components. The three main types of analytical procedure used to determine the ash content of foods are based on this principle: *dry* ashing, *wet* ashing and *low temperature plasma dry* ashing. The method chosen for a particular analysis depends on the reason for carrying out the analysis, the type of food analyzed and the equipment available. Ashing may also be used as the first step in preparing samples for analysis of specific minerals, by atomic spectroscopy or the various traditional methods described below. Ash contents of fresh foods rarely exceed 5%, although some processed foods can have ash contents as high as 12%, *e.g.*, dried beef.

4.2.1. Sample Preparation

As with all food analysis procedures it is crucial to carefully select a sample whose composition represents that of the food being analyzed and to ensure that its composition does not change significantly prior to analysis. Typically, samples of 1-10g are used in the analysis of ash content. Solid foods are finely ground and then carefully mixed to facilitate the choice of a representative sample. Before carrying out

an ash analysis, samples that are high in moisture are often dried to prevent spattering during ashing. High fat samples are usually defatted by solvent extraction, as this facilitates the release of the moisture and prevents spattering. Other possible problems include contamination of samples by minerals in grinders, glassware or crucibles which come into contact with the sample during the analysis. For the same reason, it is recommended to use deionized water when preparing samples.

4.2.2. Dry Ashing

Dry ashing procedures use a high temperature muffle furnace capable of maintaining temperatures of between 500 and 600 ^{o}C. Water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to CO_2, H_2O and N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates. Although most minerals have fairly low volatility at these high temperatures, some are volatile and may be partially lost, *e.g.,* iron, lead and mercury. If an analysis is being carried out to determine the concentration of one of these substances then it is advisable to use an alternative ashing method that uses lower temperatures.

The food sample is weighed before and after ashing to determine the concentration of ash present. The ash content can be expressed on either a *dry* or *wet* basis:

$$\%\ \text{Ash (dry basis)} = \frac{M_{ASH}}{M_{DRY}} \times 100$$

$$\%\ \text{Ash (wet basis)} = \frac{M_{ASH}}{M_{WET}} \times 100$$

where M_{ASH} refers to the mass of the ashed sample, and M_{DRY} and M_{ASH} refer to the original masses of the dried and wet samples.

There are a number of different types of crucible available for ashing food samples, including quartz, Pyrex, porcelain, steel and platinum. Selection of an appropriate crucible depends on the sample being analyzed and the furnace temperature used. The most widely used crucibles are made from porcelain because it is relatively inexpensive to purchase, can be used up to high temperatures (< 1200^{o}C) and are easy to clean. Porcelain crucibles are resistent to acids but can be corroded by alkaline samples, and therefore different types of crucible should be used to analyze this type of sample. In addition, porcelain crucibles are prone to cracking if they experience rapid temperature changes. A number of dry ashing methods have been officially recognized for the determination of the ash content of various foods (AOAC Official Methods of Analysis). Typically, a sample is held at 500-600 ^{o}C for 24 hours.

- *Advantages:* Safe, few reagents are required, many samples can be analyzed simultaneously, not labor intensive, and ash can be analyzed for specific mineral content.

- *Disadvantages:* Long time required (12-24 hours), muffle furnaces are quite costly to run due to electrical costs, loss of volatile minerals at high temperatures, *e.g.,* Cu, Fe, Pb, Hg, Ni, Zn.

Recently, analytical instruments have been developed to dry ash samples based on microwave heating. These devices can be programmed to initially remove most of the moisture (using a relatively low heat) and then convert the sample to ash (using a relatively high heat). Microwave instruments greatly reduce the time required to carry out an ash analysis, with the analysis time often being less than an hour. The major disadvantage is that it is not possible to simultaneously analyze as many samples as in a muffle furnace.

4.2.3. Wet Ashing

Wet ashing is primarily used in the preparation of samples for subsequent analysis of specific minerals (see later). It breaks down and removes the organic matrix surrounding the minerals so that they are left in an aqueous solution. A dried ground food sample is usually weighed into a flask containing strong acids and oxidizing agents (*e.g.*, nitric, perchloric and/or sulfuric acids) and then heated. Heating is continued until the organic matter is completely digested, leaving only the mineral oxides in solution. The temperature and time used depends on the type of acids and oxidizing agents used. Typically, a digestion takes from 10 minutes to a few hours at temperatures of about 350^{o}C. The resulting solution can then be analyzed for specific minerals.

- *Advantages:* Little loss of volatile minerals occurs because of the lower temperatures used, more rapid than dry ashing.
- *Disadvantages* Labor intensive, requires a special fume-cupboard if perchloric acid is used because of its hazardous nature, low sample throughput.

4.2.4. Low Temperature Plasma Ashing

A sample is placed into a glass chamber which is evacuated using a vacuum pump. A small amount of oxygen is pumped into the chamber and broken down to nascent oxygen ($O_2 \rightarrow 2O^{\cdot}$) by application of an electromagnetic radio frequency field. The organic matter in the sample is rapidly oxidized by the nascent oxygen and the moisture is evaporated because of the elevated temperatures. The relatively cool temperatures ($< 150^{o}$C) used in low-temperature plasma ashing cause less loss of volatile minerals than other methods.

- *Advantages:* Less chance of losing trace elements by volatilization
- *Disadvantages:* Relatively expensive equipment and small sample throughput.

4.2.5. Determination of Water Soluble and Insoluble Ash

As well as the total ash content, it is sometimes useful to determine the ratio of water soluble to water-insoluble ash as this gives a useful indication of the quality of certain foods, *e.g.,* the fruit content of preserves and jellies. Ash is diluted with

distilled water then heated to nearly boiling, and the resulting solution is filtered. The amount of soluble ash is determined by drying the filtrate, and the insoluble ash is determined by rinsing, drying and ashing the filter paper.

4.2.6. Comparison of Ashing Methods

The conventional dry ashing procedure is simple to carry out, is not labor intensive, requires no expensive chemicals and can be used to analyze many samples simultaneously. Nevertheless, the procedure is time-consuming and volatile minerals may be lost at the high temperatures used. Microwave instruments are capable of speeding up the process of dry ashing. Wet ashing and low temperature plasma ashing are more rapid and cause less loss of volatile minerals because samples are heated to lower temperatures. Nevertheless, the wet ashing procedure requires the use of hazardous chemicals and is labor intensive, while the plasma method requires expensive equipment and has a low sample throughput.

4.3. Determination of Specific Mineral Content

Knowledge of the concentration and type of specific minerals present in food products is often important in the food industry. The major physicochemical characteristics of minerals that are used to distinguish them from the surrounding matrix are: their low volatility; their ability to react with specific chemical reagents to give measurable changes; and their unique electromagnetic spectra. The most effective means of determining the type and concentration of specific minerals in foods is to use atomic absorption or emission spectroscopy. Instruments based on this principle can be used to quantify the entire range of minerals in foods, often to concentrations as low as a few ppm. For these reasons they have largely replaced traditional methods of mineral analysis in institutions that can afford to purchase and maintain one, or that routinely analyze large numbers of samples. Institutions that do not have the resources or sample throughput to warrant purchasing an atomic spectroscopy instrument rely on more traditional methods that require chemicals and equipment commonly found in food laboratories. Many of the minerals of importance to food scientists can be measured using one of these traditional methods.

4.3.1. Sample preparation

Many of the analytical methods used to determine the specific mineral content of foods require that the minerals be dissolved in an aqueous solution. For this reason, it is often necessary to isolate the minerals from the organic matrix surrounding them prior to the analysis. This is usually carried out by ashing a sample using one of the methods described in the previous section. It is important that the ashing procedure does not alter the mineral concentration in the food due to volatilization. Another potential source of error in mineral analysis is the presence of contaminants in the water, reagents or glassware. For this reason, ultrapure water or reagents should be used, and/or a blank should be run at the same time as the sample being analyzed. A blank uses the same glassware and reagents as the sample being analyzed and therefore should contain the same concentration of any contaminants. The concentration of minerals in the blank is then subtracted from the value determined

for the sample. Some substances can interfere with analysis of certain minerals, and should therefore be eliminated prior to the analysis or accounted for in the data interpretation. The principles of a number of the most important traditional methods for analyzing minerals are described below. Many more traditional methods can be found in the AOAC Official Methods of Analysis.

4.3.2. Gravimetric Analysis

The element to be analyzed is precipitated from solution by adding a reagent that reacts with it to form an insoluble complex with a known chemical formula. The precipitate is separated from the solution by filtration, rinsed, dried and weighed. The amount of mineral present in the original sample is determined from a knowledge of the chemical formula of the precipitate. For example, the amount of chloride in a solution can be determined by adding excess silver ions to form an insoluble silver chloride precipitate, because it is known that Cl is 24.74% of AgCl. Gravimetric procedures are only suitable for large food samples, which have relatively high concentrations of the mineral being analyzed. They are not suitable for analysis of trace elements because balances are not sensitive enough to accurately weigh the small amount of precipitate formed.

4.3.3. Colorimetric methods

These methods rely on a change in color of a reagent when it reacts with a specific mineral in solution which can be quantified by measuring the absorbance of the solution at a specific wavelength using a spectrophotometer. Colorimetric methods are used to determine the concentration of a wide variety of different minerals. Vandate is often used as a colorimetric reagent because it changes color when it reacts with minerals. For example, the phosphorous content of a sample can be determined by adding a vandate-molybdate reagent to the sample. This forms a colored complex (yellow-orange) with the phosphorous which can be quantified by measuring the absorbance of the solution at 420nm, and comparing with a calibration curve. Different reagents are also available to colorimetrically determine the concentration of other minerals.

4.3.4. Titrations

EDTA compleximetric titration

EDTA is a chemical reagent that forms strong complexes with multivalent metallic ions. The disodium salt of EDTA is usually used because it is available in high purity: Na_2H_2Y. The complexes formed by metal ions and EDTA can be represented by the following equations:

$$m^{2+} + H_2Y^{2-} \rightarrow mY^{2-} + 2H^+$$

$$m^{3+} + H_2Y^{2-} \rightarrow mY^{-} + 2H^+$$

$$m^{4+} + H_2Y^{2-} \rightarrow mY + 2H^+$$

The calcium content of foods is often determined by this method. An ashed food sample is diluted in water and then made alkaline (pH 12.5 to 13). An indicator that can form a colored complex with EDTA is then added to the solution, and the solution is titrated with EDTA. The EDTA-indicator complex is chosen to be much weaker than the EDTA-mineral complex. Consequently, as long as multivalent ions remain in the solution the EDTA forms a strong complex with them and does not react with the indicator. However, once all the mineral ions have been complexed, any additional EDTA reacts with the indicator and forms a colored complex that is used to determine the end-point of the reaction. The calcium content of a food sample is determined by comparing the volume of EDTA required to titrate it to the end-point with a calibration curve prepared for a series of solutions of known calcium concentration. If there is a mixture of different multivalent metallic ions present in a food there could be some problems in determining the concentration of a specific type of ion. It is often possible to remove interfering ions by passing the solution containing the sample through an ion-exchange column prior to analysis.

Redox reactions

Many analytical procedures are based on coupled reduction-oxidation (redox) reactions. Reduction is the gain of electrons by atoms or molecules, whereas oxidation is the removal of electrons from atoms or molecules. Any molecular species that gains electrons during the course of a reaction is said to be *reduced*, whereas any molecular species that loses electrons is said to be *oxidized*, whether or not oxygen is involved. Electrons cannot be created or destroyed in ordinary chemical reactions and so any oxidation reaction is accompanied by a reduction reaction. These coupled reactions are called *redox* reactions:

$X^n \rightarrow X^{n+1} + e^-$ (Oxidation reaction – loss of electrons)

$Y^m + e^- \rightarrow Y^{m-1}$ (Reduction reaction – gain of electrons)

$X^n + Y^m \rightarrow X^{n+1} + Y^{m-1}$ (Coupled reaction– transfer of electrons)

Analysts often design a coupled reaction system so that one of the half-reactions leads to a measurable change in the system that can be conveniently used as an end-point, *e.g.*, a color change. Thus one of the coupled reactions usually involves the mineral being analyzed (*e.g.*, X = analyte), whereas the other involves an indicator (*e.g.*, Y = indicator).

For example, permanganate ion (MnO_4^-) is a deep purple color (oxidized form), while the mangenous ion (Mn^{2+}) is a pale pink color (reduced form). Thus permanganate titrations can be used as an indicator of many redox reactions:

$MnO_4^- + 8H^+ + 5e^- \rightarrow Mn^{2+} + 4H_20$ (Reduction reaction)

(Deep Purple) (Pale Pink)

The calcium or iron content of foods can be determined by titration with a solution of potassium permanganate, the end point corresponding to the first change of the solution from pale pink to purple. The calcium or iron content is determined from the volume of permanganate solution of known molarity that is required to reach the end-point. For iron the reaction is:

$5Fe^{2+} \rightarrow 5Fe^{3+} + 5e^-$ (Oxidation reaction)

$MnO_4^- + 8H^+ + 5e^- \rightarrow Mn^{2+} + 4H_20$ (Reduction reaction)

$5Fe^{2+} + MnO_4^- + 8H^+ \rightarrow 5Fe^{3+} + Mn^{2+} + 4H_20$ (Coupled reaction)

Potassium permanganate is titrated into the aqueous solution of ashed food. While there is Fe^{2+} remaining in the food the MnO_4^- is converted to Mn^{2+} that leads to a pale pink solution. Once all of the Fe^{2+} has been converted to Fe^{3+} then the MnO_4^- remains in solution and leads to the formation of a purple color, which is the end-point.

Precipitation titrations

When at least one product of a titration reaction is an insoluble precipitate, it is referred to as a *precipitation* titration. A titrimetric method commonly used in the food industry is the *Mohr* method for chloride analysis. Silver nitrate is titrated into an aqueous solution containing the sample to be analyzed and a chromate indicator.

$AgNO_3 + NaCl \rightarrow AgCl(s) + NaNO_3$

The interaction between silver and chloride is much stronger than that between silver and chromate. The silver ion therefore reacts with the chloride ion to form AgCl, until all of the chloride ion is exhausted. Any further addition of silver nitrate leads to the formation of silver chromate, which is an insoluble orange colored solid.

$Ag^+ + Cl^- \rightarrow AgCl$ (colorless) - until all Cl^- is complexed

$2Ag^+ + CrO_4^{2-} \rightarrow Ag_2CrO_4$ (orange) - after all Cl^- is complexed

The end point of the reaction is the first hint of an orange color. The volume of silver nitrate solution (of known molarity) required to reach the endpoint is determined, and thus the concentration of chloride in solution can be calculated.

4.3.5. Ion-Selective Electrodes

The mineral content of many foods can be determined using ion-selective electrodes (ISE). These devices work on the same principle as pH meters, but the composition of the glass electrode is different so that it is sensitive to specific types of ion (rather than H^+). Special glass electrodes are commercially available to determine the concentration of K^+, Na^+, NH_4^+, Li^+, Ca^{2+} and Rb^+ in aqueous solution. Two electrodes are dipped into an aqueous solution containing the dissolved mineral: a

reference electrode and a ion-selective electrode. The voltage across the electrodes depends on the concentration of the mineral in solution and is measured at extremely low current to prevent alterations in ion concentration. The concentration of a specific mineral is determined from a calibration curve of voltage versus the logarithm of concentration. The major advantages of this method are its simplicity, speed and ease of use. The technique has been used to determine the salt concentration of butter, cheese and meat, the calcium concentration of milk and the CO_2 concentration of soft drinks. In principle, an ion selective electrode is only sensitive to one type of ion, however, there is often interference from other types of ions. This problem can often be reduced by adjusting pH, complexing or precipitating the interfering ions.

Finally, it should be noted that the ISE technique is only sensitive to the concentration of *free* ions present in a solution. If the ions are complexed with other components, such as chelating agents or biopolymers, then they will not be detected. The ISE technique is therefore particularly useful for quantifying the binding of minerals to food components. If one wants to determine the total concentration of a specific ion in a food (rather than the free concentration), then one needs to ensure that ion binding does not occur, *e.g.*, by ashing the food.

4.3.6 Atomic Spectroscopy

The determination of mineral type and concentration by atomic spectroscopy is more sensitive, specific, and quicker than traditional wet chemistry methods. For this reason it has largely replaced traditional methods in laboratories that can afford it or that routinely analyze for minerals.

Principles of Atomic Spectroscopy

The primary cause of absorption and emission of radiation in atomic spectroscopy is *electronic transitions* of outer shell electrons. Photons with the energy associated with this type of transition are found in the UV-visible part of the electromagnetic spectrum. In this respect atomic spectroscopy is similar to UV-visible spectroscopy, however, the samples used in atomic spectroscopy are individual atoms in a gaseous state, whereas those used in UV-visible spectroscopy are molecules dissolved in liquids. This has important consequences for the nature of the spectra produced. In atomic spectroscopy the peaks are narrow and well defined, but in UV-visible spectroscopy they are broad and overlap with one another. The are two major reasons for this. Firstly, because absorption or emission is from atoms, rather than molecules, there are no vibrational or rotational transitions superimposed on the electronic transitions. Secondly, because the atoms are in a gaseous state they are well separated from each other and do not interact with neighboring molecules.

The energy change associated with a transition between two energy levels is related to the wavelength of the absorbed radiation: $\Delta E = hc/\lambda$, where, h = Planks constant, c = the speed of light and λ = the wavelength. Thus for a given transition between two energy states radiation of a discrete wavelength is either absorbed or

emitted. Each element has a unique electronic structure and therefore it has a unique set of energy levels. Consequently, it absorbs or emits radiation at specific wavelengths. Each spectrum is therefore like a "fingerprint" that can be used to identify a particular element. In addition, because the absorption and emission of radiation occurs at different wavelengths for different types of atom, one element can be distinguished from others by making measurements at a wavelength where it absorbs or emits radiation, but the other elements do not.

Absorption occurs primarily when electrons in the ground state are promoted to various excited states. Emission occurs when electrons in an excited state fall back to a lower energy level. Atoms can exist in a number of different excited states, and can fall back to one of many different lower energy states (not necessarily the ground state). Thus there are many more lines in an emission spectra than there are in an absorption spectra.

Atomic spectroscopy is used to provide information about the type and concentration of minerals in foods. The type of minerals is determined by measuring the position of the peaks in the emission or absorption spectra. The concentration of mineral components is determined by measuring the intensity of a spectral line known to correspond to the particular element of interest. The reduction in intensity of an electromagnetic wave that travels through a sample is used to determine the absorbance: $A = -\log(I/I_o)$. The Beer-Lambert law can then be used to relate the absorbance to the concentration of atoms in the sample: $A = a.b.c$, where A is absorbance, a is extinction cofficient, b is sample pathlength and c is concentration of absorbing species. In practice, there are often deviations from the above equation and so it is often necessary to prepare a calibration curve using a series of standards of known concentration prepared using the same reagents as used to prepare the sample. It is also important to run a blank to take into account any impurities in the reagents that might interfere with the analysis.

Atomic Absorption Spectroscopy

Atomic absorption spectroscopy (AAS) is an analytical method that is based on the absorption of UV-visible radiation by free atoms in the gaseous state. The food sample to be analyzed is normally ashed and then dissolved in an aqueous solution. This solution is placed in the instrument where it is heated to vaporize and atomize the minerals. A beam of radiation is passed through the atomized sample, and the absorption of radiation is measured at specific wavelengths corresponding to the mineral of interest. Information about the type and concentration of minerals present is obtained by measuring the location and intensity of the peaks in the absorption spectra.

Instrumentation

The radiation source. The most commonly used source of radiation in AAS is the hollow cathode lamp. This is a hollow tube filled with argon or neon, and a cathode filament made of the metallic form of the element to be analyzed. When a voltage is applied across the electrodes, the lamp emits radiation characteristic of the metal in the cathode *i.e.*, if the cathode is made of sodium, a sodium emission spectrum is produced. When this radiation passes through a sample containing sodium atoms it

will be absorbed because it contains radiation of *exactly* the right wavelength to promote transition from one energy level to another. Thus a different lamp is needed for each type of element analyzed.

Chopper. The radiation arriving at the detector comes from two different sources: (i) radiation emitted by the filament of the lamp (which is partially absorbed by the sample); (ii) radiation that is emitted by the atoms in the sample that have been excited to higher energy levels by absorption of energy from the atomizer. To quantify the concentration of minerals in a sample using AAS it is necessary to measure the reduction in amplitude of the beam of radiation that has passed through the sample, rather than the radiation emitted by the excited sample. This can be done using a mechanical device, called a chopper, in conjunction with an electronic device that distinguishes between direct and alternating currents. The chopper is a spinning disk with a series of slits which is placed between the radiation source and the sample. The radiation from the light source is therefore continuously being switched on and off at a specific frequency, *i.e.*, it is an alternating current. On the other hand, the radiation emitted from the excited atoms in the sample is constant *i.e.*, it is direct current. The overall detected radiation is therefore the sum of a varying component and a constant component. Electronic devices are available which can separate alternating and constant current. These devices are used in AAS instruments to isolate the signal generated by the light from that emitted by the atoms in the sample.

Atomizer. Atomizers are used to convert the sample to be analyzed into individual atoms. The atomization process is achieved by exposing the sample to high temperatures, and involves three stages: (i) removal of water associated with molecules, (ii) conversion of molecules into a gas, and (iii) atomization of molecules. At higher temperatures the atoms may become ionized, which is undesirable because the atomic spectra of ionized atoms is different from that of non-ionized ones. Consequently, it is important to use a high enough temperature to atomize the molecules, but not so high that the atoms are ionized. Two types of atomizer are commonly used in atomic absorption instruments: flame and electrothermal atomization.

- Flame-atomizers consist of a nebulizer and a burner. The nebulizer converts the solution into a fine mist or aerosol. The sample is forced through a tiny hole into a chamber through which the oxidant and fuel are flowing. The oxidant and fuel carry the sample into the flame. The burner is usually 5 -10 centimeters long so as to give a long pathlength for the radiation to travel along. The characteristics of the flame can be altered by varying the relative proportions and types of oxidant and fuel used in the flame. Air-acetelyne and Nitrogen oxide-acetylene are the most commonly used mixtures of oxidant and fuel. Thus flames with different temperatures can be produced. This is important because the energy required to cause atomization, but not ionization, varies from substance to substance. Instrument manufactures provide guidelines with their instruments about the type of flame to use for specific elements.
- In electrothermal AAS the sample is placed in a small graphite cup which is electrically heated to a temperature (typically 2,000 - 3,000 ^{o}C) high enough to produce volatilization and atomization. The cup is positioned so that the radiation beam passes through the atomized sample. The advantage of

electrothermal atomizers is that smaller samples are required and detection limits are lower. Major disadvantages are that they are more expensive to purchase, have a lower sample throughput, are more difficult to operate and have a lower precision than flame-atomizers.

Wavelength selector. A wavelength selector is positioned in the optical path between the flame (or furnace) and the detector. It's purpose is to isolate the spectral line of interest from the rest of the radiation coming from the sample, so that only the radiation of the desired wavelength reaches the detector. Wavelength selectors are typically, monochromatic gratings or filters.

Detector/Readout. The detector is a photomultiplier tube that converts electromagnetic energy reaching it into an electrical signal. Most modern instruments have a computer to display the signal output and store the spectra.

Atomic Emission Spectroscopy

Atomic emission spectroscopy (AES) is different from AAS, because it utilizes the emission of radiation by a sample, rather than the absorption. For this reason samples usually have to be heated to a higher temperature so that a greater proportion of the atoms are in an excited state (although care must be taken to ensure that ionization does not occur because the spectra from ionized atoms is different from that of non-ionized atoms). There are a number of ways that the energy can be supplied to a sample, including heat, light, electricity and radio waves.

Instrumentation

In AES the sample itself acts as the source of the detected radiation, and therefore there is no need to have a separate radiation source or a chopper. The sample is heated to a temperature where it is atomized and a significant proportion of the atoms is in an excited state. Atomic emissions are produced when the electrons in an excited state fall back to lower energy levels. Since the allowed energy levels for each atom are different, they each have characteristic emission spectrum from which they can be identified. Since a food usually contains a wide variety of different minerals, each with a characteristics emission spectrum, the overall spectrum produced contains many absorption peaks. The emitted radiation is therefore passed through a wavelength selector to isolate specific peaks in the spectra corresponding to the atom of interest, and the intensity of the peak is measured using a detector and displayed on a read-out device.

Atomization-Excitation Source. The purpose of the atomization-excitation source is to atomize the sample, and to excite the atoms so that they emit a significant amount of detectable radiation. The two most commonly used forms of atomization-excitation sources in food analysis are Flame and Inductively Coupled Plasma (ICP) devices.

- In flame-AES a nebulizer-burner system is used to atomize the minerals in the sample and excite a large proportion of them to higher energy levels.
- In ICP-AES a special device is used that heats the sample to very high temperatures (6,000 to 10,000 K) in the presence of argon ions. The minerals

in the sample are not ionized at these temperatures because of the high concentration of argon ions ($Ar \Leftrightarrow Ar^+ + e^-$) leads to the release of electrons that push the equilibrium towards the non-ionized form of the mineral ($M^+ + e^- \Leftrightarrow M$).

Wavelength selectors. Wavelength selectors are used to isolate particular spectral lines, which are characteristic of the material being studied, from all the other spectral lines. A number of different types of wavelength selector are available including filters and gratings. A filter can only be used to measure the intensity at a particular fixed wavelength, whereas a grating can be used to measure the intensity at many different wavelengths. A filter can therefore only be used to analyze for one type of mineral, whereas a grating can be used to measure many different types of minerals.

Practical considerations

Prior to making atomic spectroscopy measurements a food sample is usually ashed. The resulting ash is dissolved in a suitable solvent, such as water or dilute HCl, before injecting it into the instrument. Sometimes it is possible to analyze a sample without ashing it first. For example, vegetables oils can be analyzed by dissolving them in acetone or ethanol and injecting them directly into the instrument.

Concentrations of mineral elements in foods are often at the trace level and so it is important to use very pure reagents when preparing samples for analysis. Similarly, one should ensure that glassware in very clean and dry, so that it contains no contaminating elements. It is also important to ensure there are no interfering substances in the sample whose presence would lead to erroneous results. An interfering substance could be something that absorbs at the same wavelength as the mineral being analyzed, or something that binds to the mineral and prevents it from being efficiently atomized. There are various techniques available for removing the effects of these interfering substances.

5. Analysis of Lipids

5.1. Introduction

Lipids are one of the major constituents of foods, and are important in our diet for a number of reasons. They are a major source of energy and provide essential lipid nutrients. Nevertheless, over-consumption of certain lipid components can be detrimental to our health, *e.g.* cholesterol and saturated fats. In many foods the lipid component plays a major role in determining the overall physical characteristics, such as flavor, texture, mouthfeel and appearance. For this reason, it is difficult to develop low-fat alternatives of many foods, because once the fat is removed some of the most important physical characteristics are lost. Finally, many fats are prone to lipid oxidation, which leads to the formation of off-flavors and potentially harmful products. Some of the most important properties of concern to the food analyst are*:*

- Total lipid concentration
- Type of lipids present
- Physicochemical properties of lipids, *e.g.*, crystallization, melting point, smoke point, rheology, density and color
- Structural organization of lipids within a food

5.2. Properties of Lipids in Foods

Lipids are usually defined as those components that are soluble in organic solvents (such as ether, hexane or chloroform), but are insoluble in water. This group of substances includes triacylglycercols, diacylglycercols, monoacylglycercols, free fatty acids, phospholipids, sterols, caretonoids and vitamins A and D. The lipid fraction of a fatty food therefore contains a complex mixture of different types of molecule. Even so, triacylglycercols are the major component of most foods, typically making up more than 95 to 99% of the total lipids present. Triacylglycerols are esters of three fatty acids and a glycerol molecule. The fatty acids normally found in foods vary in chain length, degree of unsaturation and position on the glycerol molecule. Consequently, the triacylglycerol fraction itself consists of a complex mixture of different types of molecules. Each type of fat has a different profile of lipids present which determines the precise nature of its nutritional and physiochemical properties. The terms fat, oil and lipid are often used interchangeably by food scientists. Although sometimes the term *fat* is used to describe those lipids that are solid at the specified temperature, whereas the term *oil* is used to describe those lipids that are liquid at the specified temperature.

5.3. Sample Selection and Preservation

As with any analytical procedure, the validity of the results depends on proper sampling and preservation of the sample prior to analysis. Ideally, the composition of the sample analyzed should represent as closely as possible that of the food from which it was taken. The sample preparation required in lipid analysis depends on the type of food being analyzed (*e.g.* meat, milk, margarine, cookie, dairy cream), the nature of the lipid component (*e.g*. volatility, susceptibility to oxidation, physical state) and the type of analytical procedure used (*e.g.* solvent extraction, non-solvent extraction or instrumental). In order, to decide the most appropriate sample preparation procedure it is necessary to have a knowledge of the physical structure and location of the principal lipids present in the food. Since each food is different it is necessary to use different procedures for each one. Official methods have been developed for specific types of foods that stipulate the precise sample preparation procedure that should be followed. In general, sample preparation should be carried out using an environment that minimizes any changes in the properties of the lipid fraction. If lipid oxidation is a problem it is important to preserve the sample by using a nitrogen atmosphere, cold temperature, low light or adding antioxidants. If the solid fat content or crystal structure is important it may be necessary to carefully control the temperature and handling of the sample.

5.4. Determination of Total Lipid Concentration

5.4.1. Introduction

It is important to be able to accurately determine the total fat content of foods for a number of reasons:

- Economic (not to give away expensive ingredients)
- Legal (to conform to standards of identity and nutritional labeling laws)
- Health (development of low fat foods)
- Quality (food properties depend on the total lipid content)
- Processing (processing conditions depend on the total lipid content)

The principle physicochemical characteristics of lipids (the "analyte") used to distinguish them from the other components in foods (the "matrix") are their solubility in organic solvents, immiscibility with water, physical characteristics (*e.g.*, relatively low density) and spectroscopic properties. The analytical techniques based on these principles can be conveniently categorized into three different types: (i) solvent extraction; (ii) non-solvent extraction and (iii) instrumental methods.

5.4.2. Solvent Extraction

The fact that lipids are soluble in organic solvents, but insoluble in water, provides the food analyst with a convenient method of separating the lipid components in foods from water soluble components, such as proteins, carbohydrates and minerals. In fact, solvent extraction techniques are one of the most commonly used methods of isolating lipids from foods and of determining the total lipid content of foods.

Sample Preparation

The preparation of a sample for solvent extraction usually involves a number of steps:

Drying sample. It is often necessary to dry samples prior to solvent extraction, because many organic solvents cannot easily penetrate into foods containing water, and therefore extraction would be inefficient.

Particle size reduction. Dried samples are usually finely ground prior to solvent extraction to produce a more homogeneous sample and to increase the surface area of lipid exposed to the solvent. Grinding is often carried out at low temperatures to reduce the tendency for lipid oxidation to occur.

Acid hydrolysis. Some foods contain lipids that are complexed with proteins (lipoproteins) or polysaccharides (glycolipids). To determine the concentration of these components it is necessary to break the bonds which hold the lipid and non-lipid components together prior to solvent extraction. Acid hydrolysis is commonly used to release bound lipids into easily extractable forms, *e.g.* a sample is digested by heating it for 1 hour in the presence of 3N HCl acid.

Solvent Selection. The *ideal* solvent for lipid extraction would completely extract all the lipid components from a food, while leaving all the other components behind. In practice, the efficiency of solvent extraction depends on the *polarity* of the lipids present compared to the polarity of the solvent. Polar lipids (such as glycolipids or phospholipids) are more soluble in polar solvents (such as alcohols), than in non-polar solvents (such as hexane). On the other hand, non-polar lipids (such as triacylglycerols) are more soluble in non-polar solvents than in polar ones. The fact that different lipids have different polarities means that it is impossible to select a single organic solvent to extract them all. Thus the total lipid content determined by solvent extraction depends on the nature of the organic solvent used to carry out the extraction: the total lipid content determined using one solvent may be different from that determined using another solvent. In addition to the above considerations, a solvent should also be inexpensive, have a relatively low boiling point (so that it can easily be removed by evaporation), be non-toxic and be nonflammable (for safety reasons). It is difficult to find a single solvent which meets all of these requirements. Ethyl ether and petroleum ether are the most commonly used solvents, but pentane and hexane are also used for some foods.

Batch Solvent Extraction

These methods are based on mixing the sample and the solvent in a suitable container, *e.g.*, a separatory funnel. The container is shaken vigorously and the organic solvent and aqueous phase are allowed to separate (either by gravity or centrifugation). The aqueous phase is then decanted off, and the concentration of lipid in the solvent is determined by evaporating the solvent and measuring the mass of lipid remaining: $\%\text{Lipid} = 100 \times (M_{\text{lipid}}/M_{\text{sample}})$. This procedure may have to be repeated a number of times to improve the efficiency of the extraction process. In this case the aqueous phase would undergo further extractions using fresh solvent, then all the solvent fractions would be collected together and the lipid determined by weighing after evaporation of solvent. The efficiency of the extraction of a particular

type of lipid by a particular type of solvent can be quantified by an equilibrium partition coefficient, $K = c_{solvent}/c_{aqueous}$, where $c_{solvent}$ and $c_{aqueous}$ are the concentration of lipid in the solvent and aqueous phase, respectively. The higher the partition coefficient the more efficient the extraction process.

Semi-Continuous Solvent Extraction

Semi-continuous solvent extraction methods are commonly used to increase the efficiency of lipid extraction from foods. The Soxhlet method is the most commonly used example of a semi-continuous method. In the Soxhlet method a sample is dried, ground into small particles and placed in a porous thimble. The thimble is placed in an extraction chamber, which is suspended above a flask containing the solvent and below a condenser. The flask is heated and the solvent evaporates and moves up into the condenser where it is converted into a liquid that trickles into the extraction chamber containing the sample. Eventually, the solvent builds up in the extraction chamber and completely surrounds the sample. The extraction chamber is designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. As the solvent passes through the sample it extracts the lipids and carries them into the flask. The lipids then remain in the flask because of their low volatility. At the end of the extraction process, which typically lasts a few hours, the flask containing the solvent and lipid is removed, the solvent is evaporated and the mass of lipid remaining is measured (M_{lipid}). The percentage of lipid in the initial sample (M_{sample}) can then be calculated: $\%Lipid = 100 \times (M_{lipid}/M_{sample})$. A number of instrument manufacturers have designed modified versions of the Soxhlet method that can be used to determine the total lipid content more easily and rapidly (*e.g.* Soxtec).

Continuous Solvent Extraction

The Goldfish method is similar to the Soxhlet method except that the extraction chamber is designed so that the solvent just trickles through the sample rather than building up around it. This reduces the amount of time required to carry out the extraction, but it has the disadvantage that *channeling* of the solvent can occur, *i.e.*, the solvent may preferentially take certain routes through the sample and therefore the extraction is inefficient. This is not a problem in the Soxhlet method because the sample is always surrounded by solvent.

Accelerated Solvent Extraction

The efficiency of solvent extraction can be increased by carrying it out at a higher temperature and pressure than are normally used. The effectiveness of a solvent at extracting lipids from a food increases as its temperature increases, but the pressure must also be increased to keep the solvent in the liquid state. This reduces the amount of solvent required to carry out the analysis, which is beneficial from a cost and environmental standpoint. Special instruments are available to carry out solvent extraction at elevated temperatures and pressures.

Supercritical Fluid Extraction

Solvent extraction can be carried out using special instruments that use supercritical carbon dioxide (rather than organic liquids) as the solvent. These instruments are finding greater use because of the cost and environmental problems associated with the usage and disposal of organic solvents. When pressurized CO_2 is heated above a certain critical temperature it becomes a supercritical fluid, which has some of the properties of a gas and some of a liquid. The fact that it behaves like a gas means that it can easily penetrate into a sample and extract the lipids, while the fact that it behaves like a fluid means that it can dissolve a large quantity of lipids (especially at higher pressures). Instruments based on this principle heat the food sample to be analyzed in a pressurized chamber and then mix supercritical CO_2 fluid with it. The CO_2 extracts the lipid, and forms a separate solvent layer, which is separated from the aqueous components. The pressure and temperature of the solvent are then reduced which causes the CO_2 to turn to a gas, leaving the lipid fraction remaining. The lipid content of a food is determined by weighing the percentage of lipid extracted from the original sample.

5.4.3. Nonsolvent Liquid Extraction Methods.

A number of liquid extraction methods do not rely on organic solvents, but use other chemicals to separate the lipids from the rest of the food. The Babcock, Gerber and Detergent methods are examples of nonsolvent liquid extraction methods for determining the lipid content of milk and some other dairy products.

Babcock Method

A specified amount of milk is accurately pipetted into a specially designed flask (the Babcock bottle). Sulfuric acid is mixed with the milk, which digests the protein, generates heat, and breaks down the fat globule membrane that surrounds the droplets, thereby releasing the fat. The sample is then centrifuged while it is hot (55-60^{o}C) which causes the liquid fat to rise into the neck of the Babcock bottle. The neck is graduated to give the amount of milk fat present in wt%. The Babcock method takes about 45 minutes to carry out, and is precise to within 0.1%. It does not determine phospholipids in milk, because they are located in the aqueous phase or at the boundary between the lipid and aqueous phases.

Gerber Method

This method is similar to the Babcock method except that a mixture of sulfuric acid and *isoamyl alcohol,* and a slightly different shaped bottle, are used. It is faster and simpler to carry out than the Babcock method. The isoamyl alcohol is used to prevent charring of the sugars by heat and sulfuric acid which can be a problem in the Babcock method since it makes it difficult to read the fat content from the graduated flask. This method is used mainly in Europe, whilst the Babcock method is used mainly in the USA. As with the Babcock method, it does not determine phospholipids.

Detergent Method

This method was developed to overcome the inconvenience and safety concerns associated with the use of highly corrosive acids. A sample is mixed with a

combination of surfactants in a Babcock bottle. The surfactants displace the fat globule membrane which surrounds the emulsion droplets in milk and causes them to coalesce and separate. The sample is centrifuged which allows the fat to move into the graduated neck of the bottle, where its concentration can then be determined.

5.4.4. Instrumental methods

The are a wide variety of different instrumental methods available for determining the total lipid content of food materials. These can be divided into three different categories according to their physicochemical principles: (i) measurement of bulk physical properties, (ii) measurement of adsorption of radiation, and (iii) measurement of scattering of radiation. Each instrumental methods has its own advantages and disadvantages, and range of foods to which it can be applied.

Measurement of bulk physical properties

- *Density:* The density of liquid oil is less than that of most other food components, and so there is a decrease in density of a food as its fat content increases. Thus the lipid content of foods can be determined by measuring their density.
- *Electrical conductivity*: The electrical conductivity of lipids is much smaller than that of aqueous substances, and so the conductivity of a food decreases as the lipid concentration increases. Measurements of the overall electrical conductivity of foods can therefore be used to determine fat contents.
- *Ultrasonic velocity:* The speed at which an ultrasonic wave travels through a material depends on the concentration of fat in a food. Thus the lipid content can be determined by measuring its ultrasonic velocity. This technique is capable of rapid, nondestructive on-line measurements of lipid content.

Measurement of adsorption of radiation

- *UV-visible:* The concentration of certain lipids can be determined by measuring the absorbance of ultraviolet-visible radiation. The lipid must usually be extracted and diluted in a suitable solvent prior to analysis, thus the technique can be quite time-consuming and labor intensive.
- *Infrared:* This method is based on the absorbance of IR energy at a wavelength of 5.73 μm due to molecular vibrations or rotations associated with fat molecules: the greater the absorbance the more fat present. IR is particularly useful for rapid and on-line analysis of lipid content once a suitable calibration curve has been developed.
- *Nuclear Magnetic Resonance***:** NMR spectroscopy is routinely used to determine the *total lipid* concentration of foods. The lipid content is determined by measuring the area under a peak in an NMR chemical shift spectra that corresponds to the lipid fraction. Lipid contents can often be determined in a few seconds without the need for any sample preparation using commercially available instruments.
- *X-ray absorption*: Lean meat absorbs X-rays more strongly than fat, thus the X-ray absorbance decreases as the lipid concentration increases. Commercial instruments have been developed which utilize this phenomenon to determine the lipid content of meat and meat products.

Measurement of scattering of radiation

- *Light scattering:* The concentration of oil droplets in dilute food emulsions can be determined using light scattering techniques because the turbidity of an emulsion is directly proportional to the concentration of oil droplets present.
- *Ultrasonic scattering:* The concentration of oil droplets in concentrated food emulsions can be determined using ultrasonic scattering techniques because the ultrasonic velocity and absorption of ultrasound by an emulsion is related to the concentration of oil droplets present.

A number of these instrumental methods have major advantages over the extraction techniques mentioned above because they are nondestructive, require little or no sample preparation, and measurements are usually rapid, precise and simple.

A major disadvantage of the techniques which rely on measurements of the bulk physical properties of foods are that a calibration curve must be prepared between the physical property of interest and the total lipid content, and this may depend on the type of lipid present and the food matrix it is contained in. In addition, these techniques can only be used to analyze foods with relatively simple compositions. In a food that contains many different components whose concentration may vary, it is difficult to disentangle the contribution that the fat makes to the overall measurement from that of the other components.

5.4.5. Comparison of Methods

Soxhlet extraction is one of the most commonly used methods for determination of total lipids in dried foods. This is mainly because it is fairly simple to use and is the officially recognized method for a wide range of fat content determinations. The main disadvantages of the technique are that a relatively dry sample is needed (to allow the solvent to penetrate), it is destructive, and it is time consuming. For high moisture content foods it is often better to use *batch solvent* or *nonsolvent* extraction techniques. Many instrumental methods are simple to operate, rapid, reproducible, require little sample preparation and are nondestructive. Nevertheless, they are often expensive to purchase and can only be used for certain types of foods, *i.e.*, where there is no interference from other components. In addition, calibration curves prepared for instrumental methods usually require that the fat content be measured using a standard method.

Extraction techniques tend to be more accurate and more generally applicable and are therefore the standard methods for official analysis of many food materials (*e.g.*, for labeling or legal requirements). Instrumental methods are most useful for rapid measurements of fat content on-line or in quality assurance laboratories of food factories where many samples must be measured rapidly.

5.5 Determination of Lipid Composition

5.5.1. Introduction

In the previous lecture analytical methods to measure total concentration of lipids in foods were discussed, without any concern about the type of lipids present. Lipids are an extremely diverse group of compounds consisting of tri-, di- and monoacylglycercols, free fatty acids, phospholipids, sterols, caretonoids and vitamins A and D. In addition, most of these sub-groups are themselves chemically complex. All triacylglycerols are esters of glycerol and three fatty acid molecules, nevertheless, the fatty acids can have different chain lengths, branching, unsaturation, and positions on the glycerol molecule. Thus even a lipid which consists of only triacylglycerols may contain a huge number of different chemical species. It is often important for food scientists to either know or to be able to specify the concentration of the different types of lipid molecules present, as well as the total lipid concentration. Some of the most important reasons for determining the type of lipids present in foods are listed below:

- *Legal.* Government regulations often demand that the amounts of saturated, unsaturated and polyunsaturated lipids, as well as the amount of cholesterol, be specified on food labels.
- *Food Quality.* Desirable physical characteristics of foods, such as appearance, flavor, mouthfeel and texture, depend on the type of lipids present.
- *Lipid oxidation.* Foods which contain high concentrations of unsaturated lipids are particularly susceptible to lipid oxidation, which can lead to the formation of undesirable off-flavors and aromas, as well as potentially toxic compounds *e.g.*, cholesterol oxides.
- *Adulteration.* Adulteration of fats and oils can be detected by measuring the type of lipids present, and comparing them with the profile expected for an unadulterated sample.
- *Food Processing.* The manufacture of many foods relies on a knowledge of the type of lipids present in order to adjust the processing conditions to their optimum values, *e.g.* temperatures, flow rates etc.

5.5.2. Sample Preparation

It is important that the sample chosen for analysis is representative of the lipids present in the original food, and that its properties are not altered prior to the analysis. Analysis of the types of lipids present in a food usually requires that the lipid be available in a fairly pure form. Thus foods which are almost entirely lipids, such as olive oil, vegetable oil or lard, can usually be analyzed with little sample preparation. Nevertheless, for many other foods it is necessary to extract and purify the lipid component prior to analysis. Lipids can sometimes be extracted by simply applying pressure to a food to squeeze out the oil, *e.g.*, some fish, nuts and seeds. For most foods, however, more rigorous extraction methods are needed, such as the solvent or nonsolvent extraction methods described in the previous lecture. Once the lipids have been separated they are often melted (if they are not liquid already) and then filtered or centrifuged to remove any extraneous matter. In addition, they are often dried to remove any residual moisture which might interfere with the analysis. As with any analytical procedure it is important not to alter the properties of the component being analyzed during the extraction process. Oxidation of unsaturated lipids can be minimized by adding antioxidants, or by flushing containers with nitrogen gas and avoiding exposure to heat and light.

5.5.3. Separation and Analysis by Chromatography

Chromatography is one of the most powerful analytical procedures for separating and analyzing the properties of lipids, especially when combined with techniques which can be used to identify the chemical structure of the peaks, *e.g.*, mass spectrometry or NMR. A chromatographic analysis involves passing a mixture of the molecules to be separated through a column that contains a matrix capable of selectively retarding the flow of the molecules. Molecules in the mixture are separated because of their differing affinities for the matrix in the column. The stronger the affinity between a specific molecule and the matrix, the more its movement is retarded, and the slower it passes through the column. Thus different molecules can be separated on the basis of the strength of their interaction with the matrix. After being separated by the column, the concentration of each of the molecules is determined as they pass by a suitable detector (*e.g.*, UV-visible, fluorescence, or flame ionization). Chromatography can be used to determine the complete profile of molecules present in a lipid. This information can be used to: calculate the amounts of saturated, unsaturated, polyunsaturated fat and cholesterol; the degree of lipid oxidation; the extent of heat or radiation damage; detect adulteration; determine the presence of antioxidants. Various forms of chromatography are available to analyze the lipids in foods, *e.g.* thin layer chromatography (TLC), gas chromatography (GC), and high pressure liquid chromatography (HPLC).

Lipid fractions by TLC

TLC is used mainly to separate and determine the concentration of different types of lipid groups in foods, *e.g.* triacylglycerols, diacylglycerols, monoacylglycerols, cholesterol, cholesterol oxides and phospholipids. A TLC plate is coated with a suitable absorbing material and placed into an appropriate solvent. A small amount of the lipid sample to be analyzed is *spotted* onto the TLC plate. With time the solvent moves up the plate due to capillary forces and separates different lipid fractions on the basis of their affinity for the absorbing material. At the end of the separation the plate is sprayed with a dye so as to make the spots visible. By comparing the distance that the spots move with standards of known composition it is possible to identify the lipids present. Spots can be scraped off and analyzed further using techniques, such as GC, NMR or mass spectrometry. This procedure is inexpensive and allows rapid analysis of lipids in fatty foods.

Fatty acid methyl esters by GC

Intact triacylglycerols and free fatty acids are not very volatile and are therefore difficult to analyze using GC (which requires that the lipids be capable of being volatized in the instrument). For this reason lipids are usually derivitized prior to analysis to increase their volatility. Triacylglycerols are first *saponified* which breaks them down to glycerol and free fatty acids, and are then *methylated*.

Triacylglycerol $\xrightarrow{CH_3OH,NaOH}$ Fatty acid methyl esters (FAMEs) + methylated glycerol

Saponification reduces the molecular weight and methylation reduces the polarity, both of which increase the volatility of the lipids. The concentration of different

volatile fatty acid methyl esters (FAMEs) present in the sample is then analyzed using GC. The FAMES are dissolved in a suitable organic solvent that is then injected into a GC injection chamber. The sample is heated in the injection chamber to volatilize the FAMES and then carried into the separating column by a heated carrier gas. As the FAMES pass through the column they are separated into a number of peaks based on differences in their molecular weights and polarities, which are quantified using a suitable detector. Determination of the total fatty acid profile allows one to calculate the type and concentration of fatty acids present in the original lipid sample.

5.5.4. Chemical Techniques

A number of chemical methods have been developed to provide information about the type of lipids present in edible fats and oils. These techniques are much cruder than chromatography techniques, because they only give information about the average properties of the lipid components present, *e.g.* the average molecular weight, degree of unsaturation or amount of acids present. Nevertheless, they are simple to perform and do not require expensive apparatus, and so they are widely used in industry and research.

Iodine Value

The iodine value (*IV*) gives a measure of the average *degree of unsaturation* of a lipid: the higher the iodine value, the greater the number of C=C double bonds. By definition the iodine value is expressed as the grams of iodine absorbed per 100g of lipid. One of the most commonly used methods for determining the iodine value of lipids is "Wijs method". The lipid to be analyzed is weighed and dissolved in a suitable organic solvent, to which a known excess of iodine chloride is added. Some of the ICl reacts with the double bonds in the unsaturated lipids, while the rest remains:

$$\text{R-CH=CH-R} + \text{ICl}_{\text{excess}} \rightarrow \text{R-CHI-CHCl-R} + \text{ICl}_{\text{remaining}}$$

The amount of ICl that has reacted is determined by measuring the amount of ICl remaining after the reaction has gone to completion ($\text{ICl}_{\text{reacted}} = \text{ICl}_{\text{excess}} - \text{ICl}_{\text{remaining}}$). The amount of ICl remaining is determined by adding excess potassium iodide to the solution to liberate iodine, and then titrating with a sodium thiosulfate ($Na_2S_2O_3$) solution in the presence of starch to determine the concentration of iodine released:

$$\text{ICl}_{\text{remaining}} + 2\text{KI} \rightarrow \text{KCl} + \text{KI} + \text{I}_2$$

$$\text{I}_2 + \text{starch} + 2\text{Na}_2\text{S}_2\text{O}_3\ (\text{blue}) \rightarrow 2\text{NaI} + \text{starch} + \text{Na}_2\text{S}_4\text{O}_6\ (\text{colorless})$$

Iodine itself has a reddish brown color, but this is often not intense enough to be used as a good indication of the end-point of the reaction. For this reason, starch is usually used as an indicator because it forms a molecular complex with the iodine that has a deep blue color. Initially, starch is added to the solution that contains the iodine and the solution goes a dark blue. Then, the solution is titrated with a sodium thiosulfate solution of known molarity. While there is any I_2 remaining in the solution it stays blue, but once all of the I_2 has been converted to I^- it turns colorless. Thus, a change

in solution appearance from blue to colorless can be used as the end-point of the titration.

The concentration of C=C in the original sample can therefore be calculated by measuring the amount of sodium thiosulfate needed to complete the titration. The higher the degree of unsaturation, the more iodine absorbed, and the higher the iodine value. The iodine value is used to obtain a measure of the average degree of unsaturation of oils, and to follow processes such as hydrogenation and oxidation that involve changes in the degree of unsaturation.

Saponification Number

The *saponification number* is a measure of the *average molecular weight* of the triacylglycerols in a sample. Saponification is the process of breaking down a neutral fat into glycerol and fatty acids by treatment with alkali:

$$\text{Triacylglycerol} + 3\ \text{KOH} \rightarrow \text{Glycerol} + 3\ \text{Fatty acid salts of potassium}$$

The saponification number is defined as the mg of KOH required to saponify one gram of fat. The lipid is first extracted and then dissolved in an ethanol solution which contains a known excess of KOH. This solution is then heated so that the reaction goes to completion. The unreacted KOH is then determined by adding an indicator and titrating the sample with HCl. The saponification number is then calculated from a knowledge of the weight of sample and the amount of KOH which reacted. The smaller the saponification number the larger the average molecular weight of the triacylglycerols present.

Acid value

The acid value is a measure of the amount of *free acids* present in a given amount of fat. The lipids are extracted from the food sample and then dissolved in an ethanol solution containing an indicator. This solution is then titrated with alkali (KOH) until a pinkish color appears. The acid value is defined as the mg of KOH necessary to neutralize the fatty acids present in 1g of lipid. The acid value may be overestimated if other acid components are present in the system, *e.g.* amino acids or acid phosphates. The acid value is often a good measure of the break down of the triacylglycrols into free fatty acids, which has an adverse effect on the quality of many lipids.

5.5.5. Instrumental Techniques

A variety of instrumental methods can also be used to provide information about lipid composition. The most powerful of these is nuclear magnetic resonance (NMR) spectroscopy. By measuring the chemical shift spectra it is possible to determine the concentration of specific types of chemical groups present, which can be used to estimate the concentration of different types of lipids. Indirect information about the average molecular weight and degree of unsaturation of the oils can be obtained by measuring physical properties, such as density or refractive index. The refractive index increases with increasing chain length and increasing unsaturation, whereas the density decreases with increasing chain length and decreasing unsaturation.

Measurements of the refractive index or density can therefore be used to monitor processes that involve a change in the composition of oils, *e.g.* hydrogenation, which decreases the degree of unsaturation.

5.6. Methods of Analyzing Lipid Oxidation in Foods

5.6.1. Introduction

Foods which contain high concentrations of unsaturated lipids are particularly susceptible to lipid oxidation. Lipid oxidation is one of the major forms of spoilage in foods, because it leads to the formation of off-flavors and potentially toxic compounds. Lipid oxidation is an extremely complex process involving numerous reactions that give rise to a variety of chemical and physical changes in lipids:

$$\text{reactants} \rightarrow \text{primary products} \rightarrow \text{secondary products}$$

$$\text{(unsaturated lipids and } O_2\text{)} \rightarrow \text{(peroxides and conjugated dienes)} \rightarrow \text{(ketones,aldehydes,alcohols,hydrocarbons)}$$

Food scientists have developed a number of methods to characterize the extent of lipid oxidation in foods, and to determine whether or not a particular lipid is susceptible to oxidation.

5.6.2. Chromatography

Chromatography is the most powerful method of monitoring lipid oxidation because it provides a detailed profile of the fatty acids and other molecules present in lipids. Valuable information about the lipid oxidation process is obtained by measuring changes in this profile with time, especially when peaks are identified using mass spectrometry or NMR. It is possible to monitor the loss of reactants (*e.g.* unsaturated lipids) and the formation of specific reaction products (*e.g.*, aldehydes, ketones or hydrocarbons) using chromatography. These measurements may be made on non-polar lipids extracted from the food, water-soluble reaction products present in the aqueous phase of a food or volatile components in the head-space of a food.

5.6.3. Oxygen Uptake

Lipid oxidation depends on the reaction between unsaturated fatty acids and oxygen. Thus it is possible to monitor the rate at which it occurs by measuring the uptake of oxygen by the sample as the reaction proceeds. Usually, the lipid is placed in a sealed container and the amount of oxygen that must be input into the container to keep the oxygen concentration in the head-space above the sample constant is measured. The more oxygen that has to be fed into the container, the faster the rate of lipid oxidation. This technique is therefore an example of a measurement of the reduction in the concentration of reactants.

5.6.4. Peroxide value

Peroxides (R-OOH) are primary reaction products formed in the initial stages of oxidation, and therefore give an indication of the progress of lipid oxidation. One of the most commonly used methods to determine peroxide value utilizes the ability of peroxides to liberate iodine from potatssium iodide. The lipid is dissolved in a suitable organic solvent and an excess of KI is added:

$$ROOH + KI_{excess} \rightarrow ROH + KOH + I_2$$

Once the reaction has gone to completion, the amount of ROOH that has reacted can be determined by measuring the amount of iodine formed. This is done by titration with sodium thiosulfate and a starch indicator:

$$I_2 + \text{starch} + 2Na_2S_2O_3 \text{ (blue)} \rightarrow 2NaI + \text{starch} + Na_2S_4O_6 \text{ (colorless)}$$

The amount of sodium thiosulfate required to titrate the reaction is related to the concentration of peroxides in the original sample (as described earlier for the iodine value). There are a number of problems with the use of peroxide value as an indication of lipid oxidation. Firstly, peroxides are primary products that are broken down in the latter stages of lipid oxidation. Thus, a low value of PV may represent either the initial or final stages of oxidation. Secondly, the results of the procedure are highly sensitive to the conditions used to carry out the experiment, and so the test must always be standardized. This technique is an example of a measurement of the increase in concentration of primary reaction products.

5.6.5. Conjugated dienes

Almost immediately after peroxides are formed, the non-conjugated double bonds (C=C-C-C=C) that are present in natural unsaturated lipids are converted to conjugated double bonds (C=C-C=C). Conjugated dienes absorb ultraviolet radiation strongly at 233nm, whereas conjugated trienes absorb at 268nm. Thus oxidation can be followed by dissolving the lipid in a suitable organic solvent and measuring the change in its absorbance with time using a UV-visible spectrophotometer. In the later stages of lipid oxidation the conjugated dienes (which are primary products) are broken down into secondary products (which do not adsorb UV-visible light strongly) which leads to a decrease in absorbance. This method is therefore only useful for monitoring the early stages of lipid oxidation. This technique is an example of a measurement of the increase in concentration of primary reaction products.

5.6.6. Thiobarbituric acid (TBA)

This is one of the most widely used tests for determining the extent of lipid oxidation. It measures the concentration of relatively polar secondary reaction products, *i.e.*, aldehydes. The lipid to be analyzed is dissolved in a suitable non-polar solvent which is contained within a flask. An aqueous solution of TBA reagent is added to the flask and the sample is shaken, which causes the polar secondary products to be dissolved in it. After shaking the aqueous phase is separated from the non-polar solvent, placed in a test-tube, and heated for 20 minutes in boiling water, which produces a pink color. The intensity of this pink color is directly related to the concentration of TBA-reactive substances in the original sample, and is determined by

measuring its absorbance at 540 nm using a UV-visible spectrophotometer. The principle source of color is the formation of a complex between TBA and *malanoaldehyde,* although some other secondary reaction products can also react with the TBA reagent. For this reason, this test is now usually referred to as the thiobarbituric acid reactive substances (TBARS) method. TBARS is an example of a measurement of the increase in concentration of secondary reaction products.

5.6.7. Accelerated Oxidation Tests

Rather than determining the extent of lipid oxidation in a particular food, it is often more important to know its *susceptibility* to oxidation. Normally, oxidation can take a long time to occur, *e.g.,* a few days to a few months, which is impractical for routine analysis. For this reason, a number of *accelerated oxidation tests* have been developed to speed up this process. These methods artificially increase the rate of lipid oxidation by exposing the lipid to heat, oxygen, metal catalysts, light or enzymes. Even so there is always some concern that the results of accelerated tests do not adequately model lipid oxidation in real systems.

A typical accelerated oxidation test is the active oxygen method (AOM). A liquid sample is held at 98 oC while air is constantly bubbled through it. Stability is expressed as hours of heating until rancidity occurs, which may be determined by detection of a rancid odor or by measuring the peroxide value. Another widely used accelerated oxidation test is the *Schaal Oven* Test. A known weight of oil is placed in an oven at a specified temperature (about 65 oC) and the time until rancidity is detected is recorded by sensory evaluation or measuring the peroxide value.

5.7. Characterization of Physicochemical Properties

5.7.1. Introduction

In addition to their nutritional importance lipids are also used in foods because of their characteristic physicochemical properties, such as mouthfeel, flavor, texture and appearance. They are also used as heat transfer agents during the preparation of other foods, *e.g.* for frying. It is therefore important for food scientists to have analytical techniques that can be used to characterize the physicochemical properties of lipids.

5.7.2. Solid Fat Content

The solid fat content (SFC) of a lipid influences many of its sensory and physical properties, such as spreadability, firmness, mouthfeel, processing and stability. Food manufacturers often measure the variation of SFC with temperature when characterizing lipids that are used in certain foods, *e.g.,* margarine and butter. The solid fat content is defined as the percentage of the total lipid that is solid at a particular temperature, *i.e.* $SFC = 100M_{solid}/M_{total}$, where M_{solid} is the mass of the lipid that is solid and M_{total} is the total mass of the lipid in the food.

A variety of methods have been developed to measure the temperature dependence of the solid fat content. The density of solid fat is higher than the density

of liquid oil, and so there is an increase in density when a fat crystallizes and a decrease when it melts. By measuring the density over a range of temperatures it is possible to determine the solid fat content - temperature profile:

$$SFC = \frac{(\rho - \rho_L)}{(\rho_S - \rho_L)} \times 100$$

where ρ is the density of the lipid at a particular temperature, and ρ_L and ρ_S are the densities of the lipid if it were completely liquid or completely solid at the same temperature. The density is usually measured by density bottles or dilatometry.

More recently, instrumental methods based on nuclear magnetic resonance (NMR) have largely replaced density measurements, because measurements are quicker and simpler to carry out (although the instrumentation is considerably more expensive). Basically, the sample is placed into an NMR instrument and a radio frequency pulse is applied to it. This induces a NMR signal in the sample, whose decay rate depends on whether the lipid is solid or liquid. The signal from the solid fat decays much more rapidly than the signal from the liquid oil and therefore it is possible to distinguish between these two components.

Techniques based on differential scanning calorimetry are also commonly used to monitor changes in SFC. These techniques measure the heat evolved or absorbed by a lipid when it crystallizes or melts. By making these measurements over a range of temperatures it is possible to determine the melting point, the total amount of lipid involved in the transition and the SFC-temperature profile.

5.7.3. Melting point

In many situations, it is not necessary to know the SFC over the whole temperature range, instead, only information about the temperature at which melting starts or ends is required. A pure triacylglycerol has a single melting point that occurs at a specific temperature. Nevertheless, foods lipids contain a wide variety of different triacylglycerols, each with their own unique melting point, and so they melt over a wide range of temperatures. Thus the "melting point" of a food lipid can be defined in a number of different ways, each corresponding to a different amount of solid fat remaining. Some of the most commonly used "melting points" are:

- *Clear point.* A small amount of fat is placed in a capillary tube and heated at a controlled rate. The temperature at which the fat completely melts and becomes transparent is called the "clear point".
- *Slip point.* A small amount of fat is placed in a capillary tube and heated at a controlled rate. The temperature at which the fat just starts to move downwards due to its weight is called the "slip point".
- *Wiley melting point.* A disc of fat is suspended in an alcohol-water mixture of similar density and is then heated at a controlled rate. The temperature at which the disc changes shape to a sphere is called the "Wiley melting point".

5.7.4. Cloud point

This gives a measure of the temperature at which crystallization begins in a liquid oil. A fat sample is heated to a temperature where all the crystals are known to have melted (*e.g.,* 130oC). The sample is then cooled at a controlled rate and the temperature at which the liquid just goes cloudy is determined. This temperature is known as the cloud point, and is the temperature where crystals begin to form and scatter light. It is often of practical importance to have an oil which does not crystallize when stored at 0oC for prolonged periods. A simple test to determine the ability of lipids to withstand cold temperatures without forming crystals, is to ascertain whether or not a sample goes cloudy when stored for 5 hours at 0oC.

5.7.5. Smoke, Flash and Fire Points

These tests give a measure of the effect of heating on the physicochemical properties of lipids. They are particularly important for selecting lipids that are going to be used at high temperatures, *e.g.* during baking or frying. The tests reflect the amount of volatile organic material in oils and fats such as free fatty acids.

- The *smoke point* is the temperature at which the sample begins to smoke when tested under specified conditions. A fat is poured into a metal container and heated at a controlled rate in an oven. The smoke point is the temperature at which a thin continuous stream of bluish smoke is first observed.
- The *flash point* is the temperature at which a flash appears at any point on the surface of the sample due to the ignition of volatile gaseous products. The fat is poured into a metal container and heated at a controlled rate, with a flame being passed over the surface of the sample at regular intervals.
- The *fire point* is the temperature at which evolution of volatiles due to the thermal decomposition of the lipids proceeds so quickly that continuous combustion occurs (a fire).

5.7.7. Rheology

The rheology of lipids is important in many food applications. Rheology is the science concerned with the *deformation* and *flow of matter*. Most rheological tests involve applying a force to a material and measuring its flow or change in shape. Many of the textural properties that people perceive when they consume foods are largely rheological in nature, *e.g.,* creaminess, juiciness, smoothness, brittleness, tenderness, hardness, *etc*. The stability and appearance of foods often depends on the rheological characteristics of their components. The flow of foods through pipes or the ease at which they can be packed into containers are also determined by their rheology. Liquid oils are usually characterized in terms of their flow properties (viscosity), whereas viscoelastic or plastic "solids" are characterized in terms of both their elastic (elastic modulus) and flow properties. A wide variety of experimental techniques are available to characterize the rheological properties of food materials.

One of the most important rheological characteristics of lipids is their "plasticity", because this determines their "spreadability". The plasticity of a lipid is due to the fact that fat crystals can form a three-dimensional network that gives the product some solid-like characteristics. Below a certain stress (known as the "yield stress") the product behaves like a solid with an elastic modulus because the crystal network is not disrupted, but above this stress it flows like a liquid because the crystal network is

continually disrupted. Rheological techniques are therefore needed to measure the change in deformation of a lipid when stresses are applied.

6. Analysis of Proteins

6.1 Introduction

Proteins are polymers of amino acids. Twenty different types of amino acids occur naturally in proteins. Proteins differ from each other according to the type, number and sequence of amino acids that make up the polypeptide backbone. As a result they have different molecular structures, nutritional attributes and physiochemical properties. Proteins are important constituents of foods for a number of different reasons. They are a major source of *energy*, as well as containing essential amino-acids, such as lysine, tryptophan, methionine, leucine, isoleucine and valine, which are essential to human health, but which the body cannot synthesize. Proteins are also the major structural components of many natural foods, often determining their overall texture, *e.g.*, tenderness of meat or fish products. Isolated proteins are often used in foods as ingredients because of their unique functional properties, *i.e.*, their ability to provide desirable appearance, texture or stability. Typically, proteins are used as gelling agents, emulsifiers, foaming agents and thickeners. Many food proteins are enzymes which are capable of enhancing the rate of certain biochemical reactions. These reactions can have either a favorable or detrimental effect on the overall properties of foods. Food analysts are interested in knowing the total concentration, type, molecular structure and functional properties of the proteins in foods.

6.2. Determination of Overall Protein Concentration

6.2.1. Kjeldahl method

The Kjeldahl method was developed in 1883 by a brewer called Johann Kjeldahl. A food is digested with a strong acid so that it releases nitrogen which can be determined by a suitable titration technique. The amount of protein present is then calculated from the nitrogen concentration of the food. The same basic approach is still used today, although a number of improvements have been made to speed up the process and to obtain more accurate measurements. It is usually considered to be *the* standard method of determining protein concentration. Because the Kjeldahl method does not measure the protein content directly a *conversion factor (F)* is needed to convert the measured nitrogen concentration to a protein concentration. A conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein) is used for many applications, however, this is only an average value, and each protein has a different conversion factor depending on its amino-acid composition. The Kjeldahl method can conveniently be divided into three steps: digestion, neutralization and titration.

6.2.1.1. Principles

Digestion

The food sample to be analyzed is weighed into a *digestion flask* and then digested by heating it in the presence of sulfuric acid (an oxidizing agent which digests the food),

anhydrous sodium sulfate (to speed up the reaction by raising the boiling point) and a catalyst, such as copper, selenium, titanium, or mercury (to speed up the reaction). Digestion converts any nitrogen in the food (other than that which is in the form of nitrates or nitrites) into ammonia, and other organic matter to $C0_2$ and H_20. Ammonia gas is not liberated in an acid solution because the ammonia is in the form of the ammonium ion (NH_4^+) which binds to the sulfate ion (SO_4^{2-}) and thus remains in solution:

$N(food) \rightarrow (NH_4)_2SO_4$ (1)

Neutralization

After the digestion has been completed the digestion flask is connected to a *recieving flask* by a tube. The solution in the digestion flask is then made alkaline by addition of sodium hydroxide, which converts the ammonium sulfate into ammonia gas:

$(NH_4)_2SO_4 + 2\ NaOH \rightarrow 2NH_3 + 2H_2O + Na_2SO_4$ (2)

The ammonia gas that is formed is liberated from the solution and moves out of the digestion flask and into the receiving flask - which contains an excess of boric acid. The low pH of the solution in the receiving flask converts the ammonia gas into the ammonium ion, and simultaneously converts the boric acid to the borate ion:

$NH_3 + H_3BO_3$ (boric acid) $\rightarrow NH_4^+ + H_2BO_3^-$ (borate ion) (3)

Titration

The nitrogen content is then estimated by titration of the ammonium borate formed with standard sulfuric or hydrochloric acid, using a suitable indicator to determine the end-point of the reaction.

$H_2BO_3^- + H^+ \rightarrow H_3BO_3$ (4)

The concentration of hydrogen ions (in moles) required to reach the end-point is equivalent to the concentration of nitrogen that was in the original food (Equation 3). The following equation can be used to determine the nitrogen concentration of a sample that weighs *m* grams using a *x*M HCl acid solution for the titration:

$$\%\,N = \frac{x\ \text{moles}}{1000\ \text{cm}^3} \times \frac{(v_s - v_b)\ \text{cm}^3}{m\ \text{g}} \times \frac{14\ \text{g}}{\text{moles}} \times 100 \quad (5)$$

Where v_s and v_b are the titration volumes of the sample and blank, and 14g is the molecular weight of nitrogen N. A blank sample is usually ran at the same time as the material being analyzed to take into account any residual nitrogen which may be in the reagents used to carry out the analysis. Once the nitrogen content has been

determined it is converted to a protein content using the appropriate conversion factor: %Protein = F× %N.

6.2.1.4. Advantages and Disadvantages

Advantages. The Kjeldahl method is widely used internationally and is still the standard method for comparison against all other methods. Its universality, high precision and good reproducibility have made it the major method for the estimation of protein in foods.

Disadvantages. It does not give a measure of the true protein, since all nitrogen in foods is not in the form of protein. Different proteins need different correction factors because they have different amino acid sequences. The use of concentrated sulfuric acid at high temperatures poses a considerable hazard, as does the use of some of the possible catalysts The technique is time consuming to carry-out.

6.2.2. Enhanced Dumas method

Recently, an automated instrumental technique has been developed which is capable of rapidly measuring the protein concentration of food samples. This technique is based on a method first described by a scientist called Dumas over a century and a half ago. It is beginning to compete with the Kjeldahl method as the standard method of analysis for proteins for some foodstuffs due to its rapidness.

6.2.2.1. General Principles

A sample of known mass is combusted in a high temperature (about 900 ^{o}C) chamber in the presence of oxygen. This leads to the release of CO_2, H_2O and N_2. The CO_2 and H_2O are removed by passing the gasses over special columns that absorb them. The nitrogen content is then measured by passing the remaining gasses through a column that has a thermal conductivity detector at the end. The column helps separate the nitrogen from any residual CO_2 and H_2O that may have remained in the gas stream. The instrument is calibrated by analyzing a material that is pure and has a known nitrogen concentration, such as EDTA (= 9.59%N). Thus the signal from the thermal conductivity detector can be converted into a nitrogen content. As with the Kjeldahl method it is necessary to convert the concentration of nitrogen in a sample to the protein content, using suitable conversion factors which depend on the precise amino acid sequence of the protein.

6.2.2.2. Advantages and Disadvantages

Advantages: It is much faster than the Kjeldahl method (under 4 minutes per measurement, compared to 1-2 hours for Kjeldahl). It doesn't need toxic chemicals or catalysts. Many samples can be measured automatically. It is easy to use.

Disadvantages: High initial cost. It does not give a measure of the true protein, since all nitrogen in foods is not in the form of protein. Different proteins need different correction factors because they have different amino acid sequences. The small sample size makes it difficult to obtain a representative sample.

6.2.3. Methods using UV-visible spectroscopy

A number of methods have been devised to measure protein concentration, which are based on UV-visible spectroscopy. These methods use either the natural ability of proteins to absorb (or scatter) light in the UV-visible region of the electromagnetic spectrum, or they chemically or physically modify proteins to make them absorb (or scatter) light in this region. The basic principle behind each of these tests is similar. First of all a calibration curve of absorbance (or turbidity) versus protein concentration is prepared using a series of protein solutions of known concentration. The absorbance (or turbidity) of the solution being analyzed is then measured at the same wavelength, and its protein concentration determined from the calibration curve. The main difference between the tests are the chemical groups which are responsible for the absorption or scattering of radiation, *e.g.*, peptide bonds, aromatic side-groups, basic groups and aggregated proteins.

A number of the most commonly used UV-visible methods for determining the protein content of foods are highlighted below:

6.2.3.1. Principles

Direct measurement at 280nm

Tryptophan and tyrosine absorb ultraviolet light strongly at 280 nm. The tryptophan and tyrosine content of many proteins remains fairly constant, and so the absorbance of protein solutions at 280nm can be used to determine their concentration. The advantages of this method are that the procedure is simple to carry out, it is nondestructive, and no special reagents are required. The major disadvantage is that nucleic acids also absorb strongly at 280 nm and could therefore interfere with the measurement of the protein if they are present in sufficient concentrations. Even so, methods have been developed to overcome this problem, *e.g.*, by measuring the absorbance at two different wavelengths.

Biuret Method

A violet-purplish color is produced when cupric ions (Cu^{2+}) interact with *peptide bonds* under alkaline conditions. The biuret reagent, which contains all the chemicals required to carry out the analysis, can be purchased commercially. It is mixed with a protein solution and then allowed to stand for 15-30 minutes before the absorbance is read at 540 nm. The major advantage of this technique is that there is no interference from materials that adsorb at lower wavelengths, and the technique is less sensitive to protein type because it utilizes absorption involving peptide bonds that are common to all proteins, rather than specific side groups. However, it has a relatively low sensitivity compared to other UV-visible methods.

Lowry Method

The Lowry method combines the biuret reagent with another reagent (the Folin-Ciocalteau phenol reagent) which reacts with *tyrosine and tryptophan* residues in

proteins. This gives a bluish color which can be read somewhere between 500 - 750 nm depending on the sensitivity required. There is a small peak around 500 nm that can be used to determine high protein concentrations and a large peak around 750 nm that can be used to determine low protein concentrations. This method is more sensitive to low concentrations of proteins than the biuret method.

Dye binding methods

A known excess of a negatively charged (anionic) dye is added to a protein solution whose pH is adjusted so that the proteins are positively charged (*i.e.* < the isoelectric point). The proteins form an insoluble complex with the dye because of the electrostatic attraction between the molecules, but the unbound dye remains soluble. The anionic dye binds to cationic groups of the basic amino acid residues (histidine, arganine and lysine) and to free amino terminal groups. The amount of unbound dye remaining in solution after the insoluble protein-dye complex has been removed (*e.g.*, by centrifugation) is determined by measuring its absorbance. The amount of protein present in the original solution is proportional to the amount of dye that bound to it: $dye_{bound} = dye_{initial} - dye_{free}$.

Turbimetric method

Protein molecules which are normally soluble in solution can be made to precipitate by the addition of certain chemicals, *e.g.*, trichloroacetic acid. Protein precipitation causes the solution to become turbid. Thus the concentration of protein can be determined by measuring the degree of turbidity.

6.2.3.2. Advantages and Disadvantages

Advantages: UV-visible techniques are fairly rapid and simple to carry out, and are sensitive to low concentrations of proteins.

Disadvantages: For most UV-visible techniques it is necessary to use dilute and transparent solutions, which contain no contaminating substances which absorb or scatter light at the same wavelength as the protein being analyzed. The need for transparent solutions means that most foods must undergo significant amounts of sample preparation before they can be analyzed, *e.g.*, homogenization, solvent extraction, centrifugation, filtration, which can be time consuming and laborious. In addition, it is sometimes difficult to quantitatively extract proteins from certain types of foods, especially after they have been processed so that the proteins become aggregated or covalently bound with other substances. In addition the absorbance depends on the type of protein analyzed (different proteins have different amino acid sequences).

6.2.4. Other Instrumental Techniques

There are a wide variety of different instrumental methods available for determining the total protein content of food materials. These can be divided into three different categories according to their physicochemical principles: (i) measurement of bulk

physical properties, (ii) measurement of adsorption of radiation, and (iii) measurement of scattering of radiation. Each instrumental methods has its own advantages and disadvantages, and range of foods to which it can be applied.

6.2.4.1. Principles

Measurement of Bulk Physical Properties

- *Density:* The density of a protein is greater than that of most other food components, and so there is an increase in density of a food as its protein content increases. Thus the protein content of foods can be determined by measuring their density.
- *Refractive index:* The refractive index of an aqueous solution increases as the protein concentration increases and therefore RI measurements can be used to determine the protein content.

Measurement of Adsorption of Radiation

- *UV-visible:* The concentration of proteins can be determined by measuring the absorbance of ultraviolet-visible radiation (see above).
- *Infrared:* Infrared techniques can be used to determine the concentration of proteins in food samples. Proteins absorb IR naturally due to characteristic vibrations (stretching and bending) of certain chemical groups along the polypeptide backbone. Measurements of the absorbance of radiation at certain wavelengths can thus be used to quantify the concentration of protein in the sample. IR is particularly useful for rapid on-line analysis of protein content. It also requires little sample preparation and is nondestructive. Its major disadvantages are its high initial cost and the need for extensive calibration.
- *Nuclear Magnetic Resonance***:** NMR spectroscopy can be used to determine the total protein concentration of foods. The protein content is determined by measuring the area under a peak in an NMR chemical shift spectra that corresponds to the protein fraction.

Measurement of Scattering of Radiation

- *Light scattering:* The concentration of protein aggregates in aqueous solution can be determined using light scattering techniques because the turbidity of a solution is directly proportional to the concentration of aggregates present.
- *Ultrasonic scattering:* The concentration of protein aggregates can also be determined using ultrasonic scattering techniques because the ultrasonic velocity and absorption of ultrasound are related to the concentration of protein aggregates present.

6.2.4.2. Advantages and Disadvantages

A number of these instrumental methods have major advantages over the other techniques mentioned above because they are nondestructive, require little or no sample preparation, and measurements are rapid and precise. A major disadvantage of the techniques which rely on measurements of the bulk physical properties of foods are that a calibration curve must be prepared between the physical property of interest

and the total protein content, and this may depend on the type of protein present and the food matrix it is contained within. In addition, the techniques based on measurements of bulk physicochemical properties can only be used to analyze foods with relatively simple compositions. In a food that contains many different components whose concentration may vary, it is difficult to disentangle the contribution that the protein makes to the overall measurement from that of the other components.

6.2.5. Comparison of methods

As food scientists we may often be in a position where we have to choose a particular technique for measuring the protein concentration of a food. How do we decide which technique is the most appropriate for our particular application ? The first thing to determine is what is the information going to be used for. If the analysis is to be carried out for official purposes, *e.g.*, legal or labeling requirements, then it is important to use an officially recognized method. The Kjeldahl method, and increasingly the Dumas method, have been officially approved for a wide range of food applications. In contrast, only a small number of applications of UV-visible spectroscopy have been officially recognized.

For quality control purposes, it is often more useful to have rapid and simple measurements of protein content and therefore IR techniques are most suitable. For fundamental studies in the laboratory, where pure proteins are often analyzed, UV-visible spectroscopic techniques are often preferred because they give rapid and reliable measurements, and are sensitive to low concentrations of protein.

Other factors which may have to be considered are the amount of sample preparation required, their sensitivity and their speed. The Kjeldahl, Dumas and IR methods require very little sample preparation. After a representative sample of the food has been selected it can usually be tested directly. On the other hand, the various UV-visible methods require extensive sample preparation prior to analysis. The protein must be extracted from the food into a dilute transparent solution, which usually involves time consuming homogenization, solvent extraction, filtration and centrifugation procedures. In addition, it may be difficult to completely isolate some proteins from foods because they are strongly bound to other components. The various techniques also have different sensitivities, *i.e.*, the lowest concentration of protein which they can detect. The UV-visible methods are the most sensitive, being able to detect protein concentrations as low as 0.001 wt%. The sensitivity of the Dumas, Kjeldahl and IR methods is somewhere around 0.1 wt%. The time required per analysis, and the number of samples which can be run simultaneously, are also important factors to consider when deciding which analytical technique to use. IR techniques are capable of rapid analysis (< 1 minute) of protein concentration once they have been calibrated. The modern instrumental Dumas method is fully automated and can measure the protein concentration of a sample in less than 5 minutes, compared to the Kjeldahl method which takes between 30 minutes and 2 hours to carry out. The various UV-visible methods range between a couple of minutes to an hour (depending on the type of dye that is used and how long it takes to react), although it does have the advantage that many samples can be run simultaneously. Nevertheless, it is usually necessary to carry out extensive sample preparation prior to analysis in order to get a transparent solution. Other factors which may be important

when selecting an appropriate technique are: the equipment available, ease of operation, the desired accuracy, and whether or not the technique is nondestructive.

6.3. Protein Separation and Characterization

In the previous lecture, techniques used to determine the total *concentration* of protein in a food were discussed. Food analysts are also often interested in the *type* of proteins present in a food because each protein has unique nutritional and physicochemical properties. Protein type is usually determined by separating and isolating the individual proteins from a complex mixture of proteins, so that they can be subsequently identified and characterized. Proteins are separated on the basis of differences in their physicochemical properties, such as size, charge, adsorption characteristics, solubility and heat-stability. The choice of an appropriate separation technique depends on a number of factors, including the reasons for carrying out the analysis, the amount of sample available, the desired purity, the equipment available, the type of proteins present and the cost. Large-scale methods are available for crude isolations of large quantities of proteins, whereas small-scale methods are available for proteins that are expensive or only available in small quantities. One of the factors that must be considered during the separation procedure is the possibility that the native three dimensional structure of the protein molecules may be altered.

A prior knowledge of the effects of environmental conditions on protein structure and interactions is extremely useful when selecting the most appropriate separation technique. Firstly, because it helps determine the most suitable conditions to use to isolate a particular protein from a mixture of proteins (*e.g.*, pH, ionic strength, solvent, temperature *etc*.), and secondly, because it may be important to choose conditions which will not adversely affect the molecular structure of the proteins.

6.3.1. Methods Based on Different Solubility Characteristics

Proteins can be separated by exploiting differences in their solubility in aqueous solutions. The solubility of a protein molecule is determined by its amino acid sequence because this determines its size, shape, hydrophobicity and electrical charge. Proteins can be selectively precipitated or solubilized by altering the pH, ionic strength, dielectric constant or temperature of a solution. These separation techniques are the most simple to use when large quantities of sample are involved, because they are relatively quick, inexpensive and are not particularly influenced by other food components. They are often used as the first step in any separation procedure because the majority of the contaminating materials can be easily removed.

Salting out

Proteins are precipitated from aqueous solutions when the salt concentration exceeds a critical level, which is known as *salting-out*, because all the water is "bound" to the salts, and is therefore not available to hydrate the proteins. Ammonium sulfate [$(NH_4)_2SO_4$] is commonly used because it has a high water-solubility, although other neutral salts may also be used, *e.g.*, NaCl or KCl. Generally a two-step procedure is used to maximize the separation efficiency. In the first step, the salt is added at a concentration just below that necessary to precipitate out the protein of interest. The solution is then centrifuged to remove any proteins that are less soluble than the

protein of interest. The salt concentration is then increased to a point just above that required to cause precipitation of the protein. This precipitates out the protein of interest (which can be separated by centrifugation), but leaves more soluble proteins in solution. The main problem with this method is that large concentrations of salt contaminate the solution, which must be removed before the protein can be resolubilzed, *e.g.*, by dialysis or ultrafiltration.

Isoelectric Precipitation

The *isoelectric point* (pI) of a protein is the pH where the net charge on the protein is zero. Proteins tend to aggregate and precipitate at their pI because there is no electrostatic repulsion keeping them apart. Proteins have different isoelectric points because of their different amino acid sequences (*i.e.*, relative numbers of anionic and cationic groups), and thus they can be separated by adjusting the pH of a solution. When the pH is adjusted to the pI of a particular protein it precipitates leaving the other proteins in solution.

Solvent Fractionation

The solubility of a protein depends on the *dielectric constant* of the solution that surrounds it because this alters the magnitude of the electrostatic interactions between charged groups. As the dielectric constant of a solution decreases the magnitude of the electrostatic interactions between charged species increases. This tends to decrease the solubility of proteins in solution because they are less ionized, and therefore the electrostatic repulsion between them is not sufficient to prevent them from aggregating. The dielectric constant of aqueous solutions can be lowered by adding water-soluble organic solvents, such as ethanol or acetone. The amount of organic solvent required to cause precipitation depends on the protein and therefore proteins can be separated on this basis. The optimum quantity of organic solvent required to precipitate a protein varies from about 5 to 60%. Solvent fractionation is usually performed at 0^{o}C or below to prevent protein denaturation caused by temperature increases that occur when organic solvents are mixed with water.

Denaturation of Contaminating Proteins

Many proteins are denatured and precipitate from solution when heated above a certain temperature or by adjusting a solution to highly acid or basic pHs. Proteins that are stable at high temperature or at extremes of pH are most easily separated by this technique because contaminating proteins can be precipitated while the protein of interest remains in solution.

6.3.2. Separation due to Different Adsorption Characteristics

Adsorption chromatography involves the separation of compounds by selective adsorption-desorption at a solid matrix that is contained within a column through which the mixture passes. Separation is based on the different affinities of different proteins for the solid matrix. *Affinity* and *ion-exchange* chromatography are the two major types of adsorption chromatography commonly used for the separation of

proteins. Separation can be carried out using either an open column or high-pressure liquid chromatography.

Ion Exchange Chromatography

Ion exchange chromatography relies on the reversible adsorption-desorption of ions in solution to a charged solid matrix or polymer network. This technique is the most commonly used chromatographic technique for protein separation. A positively charged matrix is called an *anion-exchanger* because it binds negatively charged ions (anions). A negatively charged matrix is called a cat*ion-exchanger* because it binds positively charged ions (cations). The buffer conditions (pH and ionic strength) are adjusted to favor maximum binding of the protein of interest to the ion-exchange column. Contaminating proteins bind less strongly and therefore pass more rapidly through the column. The protein of interest is then eluted using another buffer solution which favors its desorption from the column (*e.g.,* different pH or ionic strength).

Affinity Chromatography

Affinity chromatography uses a stationary phase that consists of a *ligand* covalently bound to a solid support. The ligand is a molecule that has a highly specific and unique reversible affinity for a particular protein. The sample to be analyzed is passed through the column and the protein of interest binds to the ligand, whereas the contaminating proteins pass directly through. The protein of interest is then eluted using a buffer solution which favors its desorption from the column. This technique is the most efficient means of separating an individual protein from a mixture of proteins, but it is the most expensive, because of the need to have columns with specific ligands bound to them.

Both ion-exchange and affinity chromatography are commonly used to separate proteins and amino-acids in the laboratory. They are used less commonly for commercial separations because they are not suitable for rapidly separating large volumes and are relatively expensive.

6.3.3. Separation Due to Size Differences

Proteins can also be separated according to their size. Typically, the molecular weights of proteins vary from about 10,000 to 1,000,000 daltons. In practice, separation depends on the *Stokes radius* of a protein, rather than directly on its molecular weight. The Stokes radius is the average radius that a protein has in solution, and depends on its three dimensional molecular structure. For proteins with the same molecular weight the Stokes radius increases in the following order: compact globular protein < flexible random-coil < rod-like protein.

Dialysis

Dialysis is used to separate molecules in solution by use of semipermeable membranes that permit the passage of molecules smaller than a certain size through, but prevent the passing of larger molecules. A protein solution is placed in dialysis tubing which is sealed and placed into a large volume of water or buffer which is

slowly stirred. Low molecular weight solutes flow through the bag, but the large molecular weight protein molecules remain in the bag. Dialysis is a relatively slow method, taking up to 12 hours to be completed. It is therefore most frequently used in the laboratory. Dialysis is often used to remove salt from protein solutions after they have been separated by salting-out, and to change buffers.

Ultrafiltration

A solution of protein is placed in a cell containing a *semipermeable membrane,* and pressure is applied. Smaller molecules pass through the membrane, whereas the larger molecules remain in the solution. The separation principle of this technique is therefore similar to dialysis, but because pressure is applied separation is much quicker. Semipermeable membranes with cutoff points between about 500 to 300,000 are available. That portion of the solution which is retained by the cell (large molecules) is called the *retentate,* whilst that part which passes through the membrane (small molecules) forms part of the *ultrafiltrate.* Ultrafiltration can be used to concentrate a protein solution, remove salts, exchange buffers or fractionate proteins on the basis of their size. Ultrafiltration units are used in the laboratory and on a commercial scale.

Size Exclusion Chromatography

This technique, sometimes known as gel filtration, also separates proteins according to their size. A protein solution is poured into a column which is packed with porous beads made of a cross-linked polymeric material (such as dextran or agarose). Molecules larger than the pores in the beads are excluded, and move quickly through the column, whereas the movement of molecules which enter the pores is retarded. Thus molecules are eluted off the column in order of decreasing size. Beads of different average pore size are available for separating proteins of different molecular weights. Manufacturers of these beads provide information about the molecular weight range that they are most suitable for separating. Molecular weights of unknown proteins can be determined by comparing their elution volumes Vo, with those determined using proteins of known molecular weight: a plot of elution volume versus log(*molecular weight*) should give a straight line. One problem with this method is that the molecular weight is not directly related to the Stokes radius for different shaped proteins.

6.3.4. Separation by Electrophoresis

Electrophoresis relies on differences in the migration of charged molecules in a solution when an electrical field is applied across it. It can be used to separate proteins on the basis of their size, shape or charge.

Non-denaturing Electrophoresis

In non-denaturing electrophoresis, a buffered solution of native proteins is poured onto a porous gel (usually polyacrylamide, starch or agarose) and a voltage is applied across the gel. The proteins move through the gel in a direction that depends on the

sign of their charge, and at a rate that depends on the magnitude of the charge, and the friction to their movement:

$$\text{mobility} = \frac{\text{applied voltage} \times \text{molecular charge}}{\text{molecular friction}}$$

Proteins may be positively or negatively charged in solution depending on their isoelectic points (pI) and the pH of the solution. A protein is negatively charged if the pH is above the pI, and positively charged if the pH is below the pI. The magnitude of the charge and applied voltage will determine how far proteins migrate in a certain time. The higher the voltage or the greater the charge on the protein the further it will move. The friction of a molecule is a measure of its resistance to movement through the gel and is largely determined by the relationship between the effective size of the molecule, and the size of the pores in the gel. The smaller the size of the molecule, or the larger the size of the pores in the gel, the lower the resistance and therefore the faster a molecule moves through the gel. Gels with different porosity's can be purchased from chemical suppliers, or made up in the laboratory. Smaller pores sizes are obtained by using a higher concentration of cross-linking reagent to form the gel. Gels may be contained between two parallel plates, or in cylindrical tubes. In non-denaturing electrophoresis the native proteins are separated based on a combination of their charge, size and shape.

Denaturing Electrophoresis

In *denaturing* electrophoresis proteins are separated primarily on their molecular weight. Proteins are denatured prior to analysis by mixing them with mercaptoethanol, which breaks down disulfide bonds, and *sodium dodecyl sulfate* (SDS*)*, which is an anionic surfactant that hydrophobically binds to protein molecules and causes them to unfold because of the repulsion between negatively charged surfactant head-groups. Each protein molecule binds approximately the same amount of SDS per unit length. Hence, the charge per unit length and the molecular conformation is approximately similar for all proteins. As proteins travel through a gel network they are primarily separated on the basis of their molecular weight because their movement depends on the size of the protein molecule relative to the size of the pores in the gel: smaller proteins moving more rapidly through the matrix than larger molecules. This type of electrophoresis is commonly called *sodium dodecyl sulfate -polyacrylamide gel electrophoresis,* or *SDS-PAGE.*

To determine how far proteins have moved a *tracking dye* is added to the protein solution, *e.g.,* bromophenol blue. This dye is a small charged molecule that migrates ahead of the proteins. After the electrophoresis is completed the proteins are made visible by treating the gel with a protein dye such as Coomassie Brilliant Blue or silver stain. The relative mobility of each protein band is calculated:

$$R_m = \frac{\text{distance protein moves}}{\text{distance dye moves}}$$

Electrophoresis is often used to determine the protein composition of food products. The protein is extracted from the food into solution, which is then separated using

electrophoresis. SDS-PAGE is used to determine the molecular weight of a protein by measuring *R*m, and then comparing it with a calibration curve produced using proteins of known molecular weight: a plot of log (molecular weight) against relative mobility is usually linear. Denaturing electrophoresis is more useful for determining molecular weights than non-denaturing electrophoresis, because the friction to movement does not depend on the shape or original charge of the protein molecules.

Isoelectric Focusing Electrophoresis

This technique is a modification of electrophoresis, in which proteins are separated by *charge* on a gel matrix which has a pH gradient across it. Proteins migrate to the location where the pH equals their isoelectric point and then stop moving because they are no longer charged. This methods has one of the highest resolutions of all techniques used to separate proteins. Gels are available that cover a narrow pH range (2-3 units) or a broad pH range (3-10 units) and one should therefore select a gel which is most suitable for the proteins being separated.

Two Dimensional Electrophoresis

Isoelectric focusing and SDS-PAGE can be used together to improve resolution of complex protein mixtures. Proteins are separated in one direction on the basis of *charge* using isoelectric focusing, and then in a perpendicular direction on the basis of *size* using SDS-PAGE.

6.3.5. Amino Acid Analysis

Amino acid analysis is used to determine the amino acid composition of proteins. A protein sample is first hydrolyzed (*e.g.* using a strong acid) to release the amino acids, which are then separated using chromatography, *e.g.*, ion exchange, affinity or absorption chromatography.

7. Analysis of Carbohydrates

7.1 Introduction

Carbohydrates are one of the most important components in many foods. Carbohydrates may be present as isolated molecules or they may be physically associated or chemically bound to other molecules. Individual molecules can be classified according to the number of monomers that they contain as monosaccharides, oligosaccharides or polysaccharides. Molecules in which the carbohydrates are covalently attached to proteins are known as *glycoproteins*, whereas those in which the carbohydrates are covalently attached to lipids are known as *glycolipids*. Some carbohydrates are digestible by humans and therefore provide an important source of energy, whereas others are indigestible and therefore do not provide energy. Indigestible carbohydrates form part of a group of substances known as *dietary fiber*, which also includes lignin. Consumption of significant quantities of dietary fiber has been shown to be beneficial to human nutrition, helping reduce the risk of certain types of cancer, coronary heart disease, diabetes and constipation. As well as being an important source of energy and dietary fiber, carbohydrates also contribute to the sweetness, appearence and textural characteristics of many foods. It is important to determine the type and concentration of carbohydrates in foods for a number of reasons.

- *Standards of Identity* - foods must have compositions which conform to government regulations
- *Nutritional Labeling* - to inform consumers of the nutritional content of foods
- *Detection of Adulteration* - each food type has a carbohydrate "fingerprint"
- *Food Quality* - physicochemical properties of foods such as sweetness, appearance, stability and texture depend on the type and concentration of carbohydrates present.
- *Economic* - industry doesn't want to give away expensive ingredients
- *Food Processing* - the efficiency of many food processing operations depends on the type and concentration of carbohydrates that are present

7.2. Classification of Carbohydrates

Monosaccharides

Monosaccharides are water-soluble crystalline compounds. They are aliphatic aldehydes or ketones which contain one carbonyl group and one or more hydroxyl groups. Most natural monosachharides have either five (pentoses) or six (hexoses) carbon atoms. Commonly occurring hexoses in foods are glucose, fructose and galactose, whilst commonly occurring pentoses are arabinose and xylose. The reactive centers of monosaccharides are the carbonyl and hydroxyl groups.

Oligosaccharides

These are relatively low molecular weight polymers of monosaccharides (< 20) that are covalently bonded through glycosidic linkages. Disaccharides consist of two monomers, whereas trisaccharides consist of three. Oligosaccharides containing glucose, fructose and galactose monomers are the most commonly occurring in foods.

Polysaccharides

The majority of carbohydrates found in nature are present as polysaccharides. Polysaccharides are high molecular weight polymers of monosaccharides (> 20). Polysaccharides containing all the same monosaccharides are called homopolysaccharides (*e.g.*, starch, cellulose and glycogen are formed from only glucose), whereas those which contain more than one type of monomer are known as heteropolysaccharides (*e.g.*, pectin, hemicellulose and gums).

7.3. Methods of Analysis

A large number of analytical techniques have been developed to measure the total concentration and type of carbohydrates present in foods (see *Food Analysis* by Nielssen or *Food Analysis* by Pomeranz and Meloan for more details). The carbohydrate content of a food can be determined by calculating the percent remaining after all the other components have been measured: %carbohydrates = 100 - %moisture - %protein - %lipid - %mineral. Nevertheless, this method can lead to erroneous results due to experimental errors in any of the other methods, and so it is usually better to directly measure the carbohydrate content for accurate measurements.

7.4. Monosaccharides and Oligosaccharides

7.4.1. Sample Preparation

The amount of preparation needed to prepare a sample for carbohydrate analysis depends on the nature of the food being analyzed. Aqueous solutions, such as fruit juices, syrups and honey, usually require very little preparation prior to analysis. On the other hand, many foods contain carbohydrates that are physically associated or chemically bound to other components, *e.g.*, nuts, cereals, fruit, breads and vegetables. In these foods it is usually necessary to isolate the carbohydrate from the rest of the food before it can be analyzed. The precise method of carbohydrate isolation depends on the carbohydrate type, the food matrix type and the purpose of analysis, however, there are some procedures that are common to many isolation techniques. For example, foods are usually dried under vacuum (to prevent thermal degradation), ground to a fine powder (to enhance solvent extraction) and then defatted by solvent extraction.

One of the most commonly used methods of extracting low molecular weight carbohydrates from foods is to boil a defatted sample with an 80% alcohol solution. Monosaccharides and oligosaccharides are soluble in alcoholic solutions, whereas

proteins, polysaccharides and dietary fiber are insoluble. The soluble components can be separated from the insoluble components by filtering the boiled solution and collecting the filtrate (the part which passes through the filter) and the retentante (the part retained by the filter). These two fractions can then be dried and weighed to determine their concentrations. In addition, to monosaccharides and oligosaccharides various other small molecules may also be present in the alcoholic extract that could interfere with the subsequent analysis *e.g.*, amino acids, organic acids, pigments, vitamins, minerals *etc.* It is usually necessary to remove these components prior to carrying out a carbohydrate analysis. This is commonly achieved by treating the solution with clarifying agents or by passing it through one or more ion-exchange resins.

- *Clarifying agents.* Water extracts of many foods contain substances that are colored or produce turbidity, and thus interfere with spectroscopic analysis or endpoint determinations. For this reason solutions are usually *clarified* prior to analysis. The most commonly used clarifying agents are heavy metal salts (such as lead acetate) which form insoluble complexes with interfering substances that can be removed by filtration or centrifugation. However, it is important that the clarifying agent does not precipitate any of the carbohydrates from solution as this would cause an underestimation of the carbohydrate content.
- *Ion-exchange.* Many monosaccharides and oligosaccharides are polar non-charged molecules and can therefore be separated from charged molecules by passing samples through ion-exchange columns. By using a combination of a positively and a negatively charged column it is possible to remove most charged contaminants. Non-polar molecules can be removed by passing a solution through a column with a non-polar stationary phase. Thus proteins, amino acids, organic acids, minerals and hydrophobic compounds can be separated from the carbohydrates prior to analysis.

Prior to analysis, the alcohol can be removed from the solutions by evaporation under vacuum so that an aqueous solution of sugars remains.

7.4.2. Chromatographic and Electrophoretic methods

Chromatographic methods are the most powerful analytical techniques for the analysis of the *type* and *concentration* of monosaccharides and oligosaccharides in foods. Thin layer chromatography (TLC), Gas chromatography (GC) and High Performance Liquid chromatography (HPLC) are commonly used to separate and identify carbohydrates. Carbohydrates are separated on the basis of their differential adsorption characteristics by passing the solution to be analyzed through a column. Carbohydrates can be separated on the basis of their partition coefficients, polarities or sizes, depending on the type of column used. HPLC is currently the most important chromatographic method for analyzing carbohydrates because it is capable of rapid, specific, sensitive and precise measurements. In addition, GC requires that the samples be volatile, which usually requires that they be derivitized, whereas in HPLC samples can often be analyzed directly. HPLC and GC are commonly used in conjunction with NMR or mass spectrometry so that the chemical structure of the molecules that make up the peaks can also be identified.

Carbohydrates can also be separated by electrophoresis after they have been derivitized to make them electrically charged, *e.g.*, by reaction with borates. A solution of the derivitized carbohydrates is applied to a gel and then a voltage is applied across it. The carbohydrates are then separated on the basis of their size: the smaller the size of a carbohydrate molecule, the faster it moves in an electrical field.

7.4.3. Chemical methods

A number of chemical methods used to determine monosaccharides and oligosaccharides are based on the fact that many of these substances are *reducing agents* that can react with other components to yield precipitates or colored complexes which can be quantified. The concentration of carbohydrate can be determined gravimetrically, spectrophotometrically or by titration. Non-reducing carbohydrates can be determined using the same methods if they are first hydrolyzed to make them reducing. It is possible to determine the concentration of both non-reducing and reducing sugars by carrying out an analysis for reducing sugars before and after hydrolyzation. Many different chemical methods are available for quantifying carbohydrates. Most of these can be divided into three catagories: titration, gravimetric and colorimetric. An example of each of these different types is given below.

Titration Methods

The Lane-Eynon method is an example of a tritration method of determining the concentration of reducing sugars in a sample. A burette is used to add the carbohydrate solution being analyzed to a flask containing a known amount of boiling copper sulfate solution and a methylene blue indicator. The reducing sugars in the carbohydrate solution react with the copper sulfate present in the flask. Once all the copper sulfate in solution has reacted, any further addition of reducing sugars causes the indicator to change from blue to white. The volume of sugar solution required to reach the end point is recorded. The reaction is not stoichemetric, which means that it is necessary to prepare a calibration curve by carrying out the experiment with a series of standard solutions of known carbohydrate concentration.

The disadvantages of this method are (i) the results depend on the precise reaction times, temperatures and reagent concentrations used and so these parameters must be carefully controlled; (ii) it cannot distinguish between different types of reducing sugar, and (iii) it cannot directly determine the concentration of non-reducing sugars, (iv) it is sucseptible to interference from other types of molecules that act as reducing agents..

Gravimetric Methods

The Munson and Walker method is an example of a gravimetric method of determining the concentration of reducing sugars in a sample. Carbohydrates are oxidized in the presence of heat and an excess of copper sulfate and alkaline tartrate under carefully controlled conditions which leads to the formation of a copper oxide precipitate:

$$\text{reducing sugar} + Cu^{2+} + \text{base} \rightarrow \text{oxidized sugar} + CuO_2\ \text{(precipitate)}$$

The amount of precipitate formed is directly related to the concentration of reducing sugars in the initial sample. The concentration of precipitate present can be determined gravimetrically (by filtration, drying and weighing), or titrimetrically (by redissolving the precipitate and titrating with a suitable indicator). This method suffers from the same disadvantages as the Lane-Eynon method, neverthless, it is more reproducible and accurate.

Colorimetric Methods

The Anthrone method is an example of a colorimetric method of determining the concentration of the total sugars in a sample. Sugars react with the anthrone reagent under acidic conditions to yield a blue-green color. The sample is mixed with sulfuric acid and the anthrone reagent and then boiled until the reaction is completed. The solution is then allowed to cool and its absorbance is measured at 620 nm. There is a linear relationship between the absorbance and the amount of sugar that was present in the original sample. This method determines both reducing and non-reducing sugars because of the presence of the strongly oxidizing sulfuric acid. Like the other methods it is non-stoichemetric and therefore it is necessary to prepare a calibration curve using a series of standards of known carbohydrate concentration.

The Phenol - Sulfuric Acid method is an example of a colorimetric method that is widely used to determine the total concentration of carbohydrates present in foods. A clear aqueous solution of the carbohydrates to be analyzed is placed in a test-tube, then phenol and sulfuric acid are added. The solution turns a yellow-orange color as a result of the interaction between the carbohydrates and the phenol. The absorbance at 420 nm is proportional to the carbohydrate concentration initially in the sample. The sulfuric acid causes all non-reducing sugars to be converted to reducing sugars, so that this method determines the total sugars present. This method is non-stoichemetric and so it is necessary to prepare a calibration curve using a series of standards of known carbohydrate concentration.

7.4.4. Enzymatic Methods

Analytical methods based on enzymes rely on their ability to catalyze specific reactions. These methods are rapid, highly specific and sensitive to low concentrations and are therefore ideal for determination of carbohydrates in foods. In addition, little sample preparation is usually required. Liquid foods can be tested directly, whereas solid foods have to be dissolved in water first. There are many enzyme assay kits which can be purchased commercially to carry out analysis for specific carbohydrates. Manufacturers of these kits provide detailed instructions on how to carry out the analysis. The two methods most commonly used to determine carbohydrate concentration are: (i) allowing the reaction to go to completion and measuring the concentration of the product, which is proportional to the concentration of the initial substrate; (ii). measuring the initial rate of the enzyme catalyzed reaction because the rate is proportional to the substrate concentration. Some examples of the use of enzyme methods to determine sugar concentrations in foods are given below:

D-Glucose/D-Fructose

This method uses a series of steps to determine the concentration of both glucose and fructose in a sample. First, glucose is converted to glucose-6-phosphate (G6P) by the enzyme hexakinase and ATP. Then, G6P is oxidized by $NADP^+$ in the presence of G6P-dehydrogenase (G6P-DH)

$$G6P + NADP^+ \rightarrow \text{gluconate-6-phosphate} + NADPH + H^+$$

The amount of NADPH formed is proportional to the concentration of G6P in the sample and can be measured spectrophotometrically at 340nm. The fructose concentration is then determined by converting the fructose into glucose, using another specific enzyme, and repeating the above procedure.

Maltose/Sucrose

The concentration of maltose and sucrose (disaccharides) in a sample can be determined after the concentration of glucose and fructose have been determined by the previous method. The maltose and sucrose are broken down into their constituent monosaccharides by the enzyme α–glucosidase:

$$\text{maltose} + H_2O \rightarrow 2\ \text{glucose}$$

$$\text{sucrose} + H_2O \rightarrow \text{glucose} + \text{fructose}$$

The concentrations of glucose and fructose can then be determined by the previous method. The major problem with this method is that many other oligosaccharides are also converted to monosaccharides by α-glucosidase, and it is difficult to determine precisely which oligosaccharides are present. This method is therefore useful only when one knows the type of carbohydrates present, but not their relative concentrations. Various other enzymatic methods are available for determining the concentration of other monosaccharides and oligosaccharides, *e.g.*, lactose, galactose and raffinose (see *Food Analysis* Nielssen).

7.4.5. Physical Methods

Many different physical methods have been used to determine the carbohydrate concentration of foods. These methods rely on their being a change in some physicochemical characteristic of a food as its carbohydrate concentration varies. Commonly used methods include polarimetry, refractive index, IR, and density.

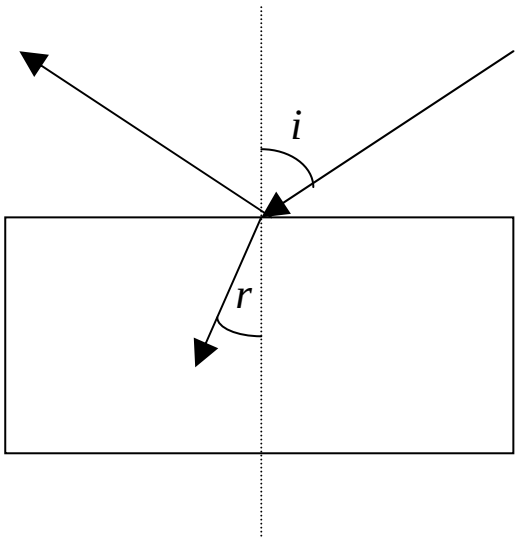
Polarimetry

Molecules that contain an asymmetric carbon atom have the ability to rotate plane polarized light. A polarimeter is a device that measures the angle that plane polarized light is rotated on passing through a solution. A polarimeter consists of a source of monochromatic light, a polarizer, a sample cell of known length, and an analyzer to measure the angle of rotation. The extent of polarization is related to the concentration of the optically active molecules in solution by the equation $\alpha = [\alpha]lc$, where α is the measured angle of rotation, $[\alpha]$ is the optical activity (which is a constant for each type of molecule), l is the pathlength and c is the concentration. The overall angle of

rotation depends on the temperature and wavelength of light used and so these parameters are usually standardized to 20^{o}C and 589.3 nm (the D-line for sodium). A calibration curve of α versus concentration is prepared using a series of solutions with known concentration, or the value of [α] is taken from the literature if the type of carbohydrates present is known. The concentration of carbohydrate in an unknown sample is then determined by measuring its angle of rotation and comparing it with the calibration curve.

Refractive Index

The refractive index (n) of a material is the velocity of light in a vacuum divided by the velocity of light in the material ($n = c/c_m$). The refractive index of a material can be determined by measuring the angle of refraction (r) and angle of incidence (i) at a boundary between it and another material of known refractive index (Snell's Law: $\sin(i)/\sin(r) = n_2/n_1$). In practice, the refractive index of carbohydrate solutions is usually measured at a boundary with quartz. The refractive index of a carbohydrate solution increases with increasing concentration and so can be used to measure the amount of carbohydrate present. The RI is also temperature and wavelength dependent and so measurements are usually made at a specific temperature (20 ^{o}C) and wavelength (589.3nm). This method is quick and simple to carry out and can be performed with simple hand-held instruments. It is used routinely in industry to determine sugar concentrations of syrups, honey, molasses, tomato products and jams.



Density

The density of a material is its mass divided by its volume. The density of aqueous solutions increases as the carbohydrate concentration increases. Thus the carbohydrate concentration can be determined by measuring density, *e.g.*, using density bottles or hydrometers. This technique is routinely used in industry for determination of carbohydrate concentrations of juices and beverages.

Infrared

A material absorbs infrared due to vibration or rotation of molecular groups. Carbohydrates contain molecular groups that absorb infrared radiation at wavelengths where none of the other major food constituents absorb consequently their concentration can be determined by measuring the infrared absorbance at these wavelengths. By carrying out measurements at a number of different specific wavelengths it is possible to simultaneously determine the concentration of carbohydrates, proteins, moisture and lipids. Measurements are normally carried out by measuring the intensity of an infrared wave reflected from the surface of a sample: the greater the absorbance, the lower the reflectance. Analytical instruments based on infrared absorbance are non-destructive and capable of rapid measurements and are therefore particularly suitable for on-line analysis or for use in a quality control laboratory where many samples are analyzed routinely.

More sophisticated instrumental methods are capable of providing information about the molecular structure of carbohydrates as well as their concentration, *e.g.*, NMR or mass spectrometry.

7.4.6. Immunoassays

Immuoassays are finding increasing use in the food industry for the qualitative and quantitative analysis of food products. Immunoassays specific for low molecular weight carbohydrates are developed by attaching the carbohydrate of interest to a protein, and then injecting it into an animal. With time the animal develops antibodies specific for the carbohydrate molecule. These antibodies can then be extracted from the animal and used as part of a test kit for determining the concentration of the specific carbohydrate in foods. Immuoassays are extremely sensitive, specific, easy to use and rapid.

7.5 Analysis of Polysaccharides and Fiber

A wide variety of polysaccharides occur in foods. Polysaccharides can be classified according to their molecular characteristics (*e.g.*, type, number, bonding and sequence of monosaccharides), physicochemical characteristics (*e.g.*, water solubility, viscosity, surface activity) and nutritional function (*e.g.*, digestible or non-digestible). Most polysaccharides contain somewhere between 100 and several thousand monosaccharides. Some polysaccharides contain all the same kind of monosaccharide (homopolysaccharides), whereas others contain a mixture of different kinds of monosaccharide (heteropolysaccharides). Some polysaccharides exist as linear chains, whereas others exist as branched chains. Some polysaccharides can be digested by human beings and therefore form an important source of energy (*e.g.*, starch), whereas others are indigestible (*e.g.*, cellulose, hemicellulose and pectins). These indigestible polysaccharides form part of a group of substances known as *dietary fiber*, which also includes lignin (which is a polymer of aromatic molecules). Consumption of many types of dietary fiber has been shown to have beneficial physiologically functional properties for humans, *e.g.*, prevention of cancer, heart disease and diabetes.

7.5.1. Analysis of Starch

Starch is the most common digestible polysaccharide found in foods, and is therefore a major source of energy in our diets. In its natural form starch exists as water-insoluble granules (3 - 60 μm), but in many processed foods the starch is no longer in this form because of the processing treatments involved (*e.g.*, heating). It consists of a mixture of two glucose homopolysaccharides: *amylose* (500-2000 glucose units) which is linear, and *amylopectin* (>1,000,000 glucose units) which is extensively branched. These two kinds of starch have different physiochemical properties and so it is often important to determine the concentration of each individual component of the starch, as well as the overall starch concentration.

Sample preparation. The starch content of most foods cannot be determined directly because the starch is contained within a structurally and chemically complex food matrix. In particular, starch is often present in a semi-crystalline form (granular or retrograded starch) that is inaccessible to the chemical reagents used to determine its concentration. It is therefore necessary to isolate starch from the other components present in the food matrix prior to carrying out a starch analysis.

In natural foods, such as legumes, cereals or tubers, the starch granules are usually separated from the other major components by drying, grinding, steeping in water, filtration and centrifugation. The starch granules are water-insoluble and have a relatively high density (1500 kg/m^3) so that they will tend to move to the bottom of a container during centrifugation, where they can be separated from the other water-soluble and less dense materials. Processed food samples are normally dried, ground and then dispersed in hot 80% ethanol solutions. The monosaccharides and oligosaccharides are soluble in the ethanol solution, while the starch is insoluble. Hence, the starch can be separated from the sugars by filtering or centrifuging the solution. If any semi-crystalline starch is present, the sample can be dispersed in water and heated to a temperature where the starch gelatinizes (> 65 ^oC). Addition of perchloric acid or calcium chloride to the water prior to heating facilitates the solubilization of starches that are difficult to extract.

Analysis methods. Once the starch has been extracted there are a number of ways to determine its concentration:

- Specific enzymes are added to the starch solution to breakdown the starch to glucose. The glucose concentration is then analyzed using methods described previously (*e.g.*, chromatography or enzymatic methods). The starch concentration is calculated from the glucose concentration.
- Iodine can be added to the starch solution to form an insoluble starch-iodine complex that can be determined *gravimetrically* by collecting, drying and weighing the precipitate formed or *titrimetrically* by determining the amount of iodine required to precipitate the starch.
- If there are no other components present in the solution that would interfere with the analysis, then the starch concentration could be determined using physical methods, *e.g.*, density, refractive index or polarimetry.

The amylose and amylopectin concentrations in a sample can be determined using the same methods as described for starch once the amylose has been separated from the amylopectin. This can be achieved by adding chemicals that form an insoluble complex with one of the components, but not with the other, *e.g.* some alcohols

precipitate amylose but not amylopectin. Some of the methods mentioned will not determine the concentration of resistant starch present in the sample. If the concentration of resistant starch is required then an additional step can be added to the procedure where dimethylsulfoxide (DMSO) is added to dissolve the resistant starch prior to carrying out the analysis.

7.5.2. Analysis of Fibers

Over the past twenty years or so nutritionists have become aware of the importance of fiber in the diet. Liberal consumption of fiber helps protect against colon cancer, cardiovascular disease and constipation. Adequate intake of dietary fiber is therefore beneficial to good health. Dietary fiber is defined as plant polysaccharides that are indigestible by humans, plus lignin. The major components of dietary fiber are cellulose, hemicellulose, pectin, hydrocolloids and lignin. Some types of starch, known as *resistant starch*, are also indigestible by human beings and may be analyzed as dietary fiber. The basis of many fiber analysis techniques is therefore to develop a procedure that mimics the processes that occur in the human digestive system.

7.5.2.1. Major Components of Dietary Fiber

Cell Wall Polysaccharides

Cellulose occurs in all plants as the principal structural component of the cell walls, and is usually associated with various hemicelluloses and lignin. The type and extent of these associations determines the characteristic textural properties of many edible plant materials. Cellulose is a long linear homopolysaccahride of glucose, typically having up to 10,000 glucose subunits. Cellulose molecules aggregate to form microfibrils that provide strength and rigidity in plant cell walls. Hemicelluloses are a heterogeneous group of branched heteropolysaccharides that contain a number of different sugars in their backbone and side-chains. By definition hemicelluloses are soluble in dilute alkali solutions, but insoluble in water. Pectins are another form of heteropolysaccharides found in cell walls that are rich in uronic acids, soluble in hot water and that are capable of forming gels.

Non Cell Wall Polysaccharides

This group of substances are also indigestible carbohydrates, but they are not derived from the cell walls of plants. Non-cell wall polysaccharides include hydrocolloids such as guar and locust bean gum, gum arabic, agar, alginates and caragenans which are commonly used in foods as gelling agents, stabilizers and thickeners.

Lignin

Lignin is a non-carbohydrate polymer that consists of about 40 aromatic subunits which are covalently linked. It is usually associated with cellulose and hemicelluloses in plant cell-walls.

7.5.2.2. Common Procedures in Sample Preparation and Analysis

There are a number of procedures that are commonly used in many of the methods for dietary fiber analysis:

- *Lipid removal.* The food sample to be analyzed is therefore dried, ground to a fine powder and then the lipids are removed by solvent extraction.
- *Protein removal.* Proteins are usually broken down and solubilized using enzymes, strong acid or strong alkali solutions. The resulting amino acids are then separated from insoluble fiber by filtration or from total fiber by selective precipitation of the fiber with ethanol solutions.
- *Starch removal.* Semi-crystalline starch is gelatinized by heating in the presence of water, and then the starch is broken down and solubilized by specific enzymes, strong acid or strong alkali. The glucose is then separated from insoluble fiber by filtration or separated from total fiber by selective precipitation of the fiber with ethanol solutions.
- *Selective precipitation of fibers.* Dietary fibers can be separated from other components in aqueous solutions by adding different concentrations of ethanol to cause selective precipitation. The solubility of monosaccharides, oligosaccharides and polysaccharides depends on the ethanol concentration. *Water*: monosaccharides, oligosaccharides, some polysaccharides and amino acids are soluble; other polysaccharides and fiber are insoluble. *80% ethanol solutions:* monosaccharides, oligosaccharides and amino acids are soluble; polysaccharides and fibers are insoluble. For this reason, *concentrated ethanol solutions* are often used to selectively precipitate fibers from other components.
- *Fiber analysis.* The fiber content of a food can be determined either *gravimetrically* by weighing the mass of an insoluble fiber fraction isolated from a sample or *chemically* by breaking down the fiber into its constituent monosaccharides and measuring their concentration using the methods described previously.

7.5.2.3. Gravimetric Methods

Crude Fiber Method

The crude fiber method gives an estimate of indigestible fiber in foods. It is determined by sequential extraction of a defatted sample with 1.25% H_2SO_4 and 1.25% NaOH. The insoluble residue is collected by filtration, dried, weighed and ashed to correct for mineral contamination of the fiber residue. Crude fiber measures cellulose and lignin in the sample, but does not determine hemicelluloses, pectins and hydrocolloids, because they are digested by the alkali and acid and are therefore not collected. For this reason many food scientists believe that its use should be discontinued. Nevertheless, it is a fairly simple method to carry out and is the official AOAC method for a number of different foodstuffs.

Total, insoluble and soluble fiber method

The basic principle of this method is to isolate the fraction of interest by selective precipitation and then to determine its mass by weighing. A gelatinized sample of dry, defatted food is enzymatically digested with α–amylase, amyloglucosidase and protease to break down the starch and protein components. The total fiber content of the sample is determined by adding 95% ethanol to the solution to precipitate all the fiber. The solution is then filtered and the fiber is collected, dried and weighed. Alternatively, the water-soluble and water-insoluble fiber components can be determined by filtering the enzymatically digested sample. This leaves the *soluble* fiber in the filtrate solution, and the *insoluble* fiber trapped in the filter. The insoluble component is collected from the filter, dried and weighed. The soluble component is precipitated from solution by adding 95% alcohol to the filtrate, and is then collected by filtration, dried and weighed. The protein and ash content of the various fractions are determined so as to correct for any of these substances which might remain in the fiber: Fiber = residue weight - weight of (protein + ash).

This method has been officially sanctioned by the AOAC and is widely used in the food industry to determine the fiber content of a variety of foods. Its main disadvantage is that it tends to overestimate the fiber content of foods containing high concentrations of simple sugars, *e.g.*, dried fruits, possibly because they get trapped in the precipitates formed when the ethanol is added.

7.5.2.4. Chemical Methods

In chemical methods, the fiber content is equal to the sum of all nonstarch monosaccharides plus lignin remaining once all the digestible carbohydrates have been removed. Monosaccharides are measured using the various methods described previously.

Englyst-Cummings Procedure

A defatted food sample is heated in water to gelatinize the starch. Enzymes are then added to digest the starch and proteins. Pure ethanol is added to the solution to precipitate the fiber, which is separated from the digest by centrifugation, and is then washed and dried. The fiber is then hydrolyzed using a concentrated sulfuric acid solution to break it down into its constituent monosaccharides, whose concentration is determined using the methods described previously, *e.g.*, colorimetrically or chromatographically. The mass of fiber in the original sample is assumed to be equal to the total mass of monosaccharides present. The concentration of insoluble and soluble dietary fiber can also be determined by this method, using similar separation steps as for the *total, insoluble and soluble* gravimetric method mentioned above.

This method can be used to determine the total, soluble and insoluble fiber contents of foods, but does not provide information about the *lignin* content. This is because lignin is not a polysaccharide, and so it is not broken down to monosaccharides during the acid digestion. For most foods this is not a problem because they have low lignin concentrations anyway. If a food does contain significant amounts of lignin then another method should be used, *e.g.*, the

gravimetric method or more sophisticated chemical methods (*e.g.*, the Theander-Marlett method).