CHAPTER 1

Methodology and Proximate Analysis

1 Extraction of Organic Analytes

Opening Statement

The preparation for analysis of a small amount of material (the sample), representative of the bulk food, and normally supplied to the analyst, can be divided into the following stages:

1. The first stage for most food matrices is to prepare a weighed and calibrated aliquot – the sample for analysis – in preparation for quantitative extraction of the compounds of interest (analytes).
2. For some food samples, the material has to be rendered accessible to the extracting agent – preparation for analysis.
3. The next stage can then be either (a) removal of the analytes from the sample matrix or (b) removal of interferents from the matrix – the extraction. In each case, the analytes are in a form to be recognised and quantified unambiguously in subsequent examinations.
4. The final stage is to examine the extract, which will normally contain matrix components other than the target analytes, using various of chemical and physical methods to make qualitative or quantitative measurements of the analytes – the analysis.

One contemporary objective in the development of analytical methodology is to automate the whole assay, and there are two ways forward. The classical extraction procedure can be given over to robotic control, or the information about the chemical composition can be "extracted" directly from the sample matrix by remote sensing. Perversely, remote sensing makes extraction redundant. Thus, it is necessary at the outset to recognise that the future analysis may be an extractionless, remote sensing, robotic operation. Although considerable

progress has been made already towards these goals, as a "hands on instrumental analytical chemist" the *modus vivendi* for this monograph was to present classical and modern experiential and methodological data in ways that may be a helpful record and also serve as a transitional reference of methodology to facilitate the advancement of the era of robotic analytical workstations.

Food Sample for Analysis

Foods (and drinks) are nutrient-containing substances that can be metabolised into body tissue and into energy to sustain body tissue. In modern parlance foods are largely solid, and drinks are largely liquid. It is convenient to refer to all nutrient sources as food – the nutrient-carrying matrix – and to consider the removal of compounds from a sample of food as an extraction. However, the English language has many words to express the idea of removing something from the whole. In analytical chemistry, for example, there is no clear distinction between a separation method and an extraction method, and it gets worse because chemists also fractionate, purify, isolate, partition, disperse and distribute components of mixtures. Here, an extraction is thought of as an operation on a sample of food that concentrates the target components, normally by removing them from the bulk of the food sample, often in preparation for further examination such as chromatographic separation. In analytical chemistry, a separation is seldom carried out on the raw material (however, see Chapter 8, Section 2, direct injection), but on an extracted or cleaned up sample for analysis. In addition, there are many procedures associated with extraction that in themselves do not actually remove anything from the sample. These processes are dealt with in Chapter 2 (Sample Preparation for Extraction), and are treated as extraction aids.

The natural origins of human foods are biologically diverse, ranging widely in texture and composition – from nutmeg to oysters. The extremely complex endogenous composition of food is made even more complex in the modern environment where so many extrinsic, additional items – additives such as antioxidants, contaminants from agriculture such as herbicides and industrial adulterants such as hydrocarbons from petroleum – may also be present. This extends the quantitative range of analyses practised by food analysts from the gram amounts encountered in proximate analysis (Section 6) to picogram and even lower amounts of highly toxic contaminants *e.g.* PCBs. To cover more than 12 orders of magnitude requires an enormously diverse armoury of techniques.

Analysis of Foods

It is usually a concern over the chemical composition or contamination of food and the effect this has on its value to the consumer that generates the need for analysis. The quality of food is based on the natural composition, the balance between the nutrient and the anti-nutrient composition. The health and prosperity of early civilisations depended upon their ability to refine their food

supply in the short term by removing toxic materials using extraction methods, or in the long term through crop selection and plant breeding.

History of Food Extraction

Many extraction methods were invented to remove sufficient quantities of toxins (anti-nutrients) from the biological source to make the material acceptable and safe to eat. Notably, nature historically used toxins in sources of human and animal foods to maintain the balance between the survival of the browser and the browsed! These practices were incorporated into the culture of the technology employed in the early analytical laboratories.

The natural processes used to extract moisture in order to increase the "shelf-life" of food and the early uses of extraction methods to concentrate important components, *e.g.* essential oils, formed the bases for methods of analysis as the science of measurement began to develop. Historically, the extraction of bulk components from food made use of physical processes, such as pressure, to remove the juice or oil from the pulverised pulp. Warm air or sun drying of tomatoes or fish extracted sufficient water to reduce bacterial attack to an acceptable level in preparation for storage. Solvent extracts of essential oils from the pulverised plant, seed, or nut were concentrated by distillation in simple stills. Spicy and resinous plants were solvent refluxed in fractionation columns and valuable components separated and extracted in this way. The modern method of supercritical fluid extraction (SFE) uses ultrapure carbon dioxide as solvent, thus eliminating the fear of toxic residues in the extract. Cold-pressing methods are still used to produce high quality extracts of citrus fruits, and hydrodistillation, the steam distillation of an aqueous solution of the food matrix, was practised, especially on powders, from earliest times. There are many other examples where extraction from the bulk material was used to refine our food supplies.

Analytical data defining food quality and the methods used to obtain them have to be validated; several regulatory bodies oversee this process (FDA, FSA, AOAC, FAO, WHO, *etc.*). In 1963, the FAO and the WHO set up the Codex Alimentarius Commission to develop food standards, guidelines and codes of practice. Their web-site is Codex@fao.org. Ultimately, there are definitive methods that can be applied to analysis that provide an acceptable degree of confidence and that are universally recognised. For example, The Canadian Health Protection Branch, Health Canada published *The Compendium of Methods for Chemical Analysis of Foods*, which was extant till 1995 and is currently being updated. The e-mail contact is Xu-Liang Cao@hc-sc.gc.ca. Details of the extraction from the food matrix of the compounds of interest will be given in the method. These data are already correlated and readily available to the practising food analyst and only illustrative examples will be further discussed. It is the historical context, background principles, general practice, and the development of emerging and tentative experimental methods leading to the ultimate automated assay that form the major part of this study.

The ways in which the methodology has been used are illustrated in the examples from the scientific literature chosen to cover a range of commodities and analytes. Modern databases contain huge amounts of information on extraction methods for food analysis and the reader may wish to base further searches for information on keywords found in the appropriate sections of this manual of methods.

Stages in Food Analysis

Stages that may be required in the analysis of foods are:

1. Setting the protocol.
2. Sampling the food.
3. Preparing the sample in readiness for extraction of the chosen analyte or compound of interest (COI), including standardisation.
4. Extraction of the COI.
5. Separation from, or removal of, substances interfering with the detection of the COI in the extract.
6. Detection (recognition or visualisation) of the COI.
7. Identification and/or quantification of the COI.
8. Recording the information.

Items 3–5 are the subjects of this monograph.

Defining the Stages in Food Analysis

Protocol. It is important to have a clearly defined protocol and to adhere to it, so that the analysis can be reported unambiguously, verified by the analyst, and, if necessary, reproduced for verification by other analysts.

Sampling. This is the process of preparing a representative portion of the whole food for analysis. This sample is usually re-sampled by the analyst (the sample for analysis) and may need treatment before the target compound(s) can be extracted. If quantitative results are required, an internal standard (*e.g.* an isomer with similar chemical properties, but distinguishable from the analyte by GC retention time, or an isotope distinguishable by its mass spectrum) may be added to allow any subsequent losses to be compensated for during the analysis.

Preparation of the Sample for Extraction. The definition of sample preparation is ambiguous in the literature, often covering all processes up to and including the separation stage. The definition of sample preparation for extraction here is "the execution of procedures necessary to prepare the original sample for extraction." Such processes include grinding, digestion, and centrifugation. Occasionally, mainly for liquid foods, no preparation for extraction is required.

Extraction. There can be no hard and fast rule, but having entitled this monograph "extraction methods" the definition of an extraction process used in collecting together relevant subjects was "one where a part of the sample is removed from the whole starting sample." It may be the part containing the compounds of interest or it may be an unwanted part being discarded, leaving a less complex and usually more concentrated remainder for further study.

Direct Analysis without Extraction. The analyte can be in sufficient concentration, and free from interference from the matrix, usually in a liquid food, that no extraction stage is necessary. For example, a sample of the matrix can be injected directly into the separation stage, *e.g.* HPLC. This possibility becomes more feasible with the use of guard columns and as the resolving power of the separation and detection stages improve. The use of chromatography–MS and electrophoresis–MS, especially MS^n and HRMS methods means that many potential interferents can be circumvented without the need to remove them by extraction. Alternatively, a colorimetric reaction can visualise and quantify the analyte in a crude extract, as is the case when the biuret reaction is used to measure protein content.

Separation. The term separation is reserved for chromatographic and electrophoretic processes where the main objective is not to remove or extract something for further stages of analysis, but to finally resolve components of mixtures for detection and identification.

Exceptions are made in the case of preparative separation, which was the forerunner of two-dimensional separation, where the fractions are collected for manual transfer to a further stage of analysis. This can be seen as a preliminary extraction – and again with multistage chromatography, where each stage serves to fractionate the mixture, presenting one or every fraction extracted as the input to further stages of analysis. Multistage or two-dimensional chromatography is capable of extremely complex, on-line, automated separations and can be seen as a combined extraction and separation system.

Because the separation stage is not in the remit for this monograph, a more pedantic stance has been taken to draw a distinction between the two stages than is necessary when dealing with the analytical process *in toto*. Nevertheless, on several occasions the separation process has been viewed as a micropreparation process simply to raise the prospects for automated microanalytical methods to be developed.

Detection for Identification and/or Quantification. The signal recorded when a component of the sample is registered (recognised, standardised or calibrated) above a base line, and the signal content is converted into qualitative or quantitative information.

Direct Detection by Remote Sensing of the Food Sample. If the analyte can be recognised (detected) in the food sample prepared for analysis by a sensing

probe, then the required analytical data can be extracted directly without the need for physical, chemical, or biochemical intervention. This can be considered to be "virtual extraction or virtual removal of interference." Such methods have the potential to be rapid, economical in time and resources, and ideal for automation.

Recording the Information. For those wishing to refresh on recording and general good analytical chemical practice, a text such as *Fundamentals of Analytical Chemistry* by D.A. Skoog and D.M. West[1] provides a thorough treatment. Some further discussion of the stages of food analysis with relevance to the extraction techniques that form the major part of the work will follow.

2 Sampling

Introduction

Assuming that the strategic arguments have been addressed and the reason for undertaking the analysis defined, the first analytical procedure is to obtain a representative sample of the bulk material. If the sampling process is inaccurate, all the subsequent, and often expensive extraction, separation and identification stages will be in jeopardy. Sampling is not covered in this monograph, but a few general comments will serve to put into perspective the material upon which the extraction will be made.

There are several different problems that can beset the sampling for analysis procedure. For example, if a new cultivar of broccoli has been created in the greenhouse on a limited experimental scale, and there are only one or two small florets on each plant, it may be permissible only to use a small part of it for, say, glucosinolate analysis. Therefore, high efficiency is required of the extraction and high sensitivity of the analytical methods employed. Even so, the sample represents only a single plant and the results should not be expressed otherwise.

A sampling problem of a different kind is generated when it is necessary to choose a representative amount from a 30-ton truck loaded with carrots from the field; how does one ensure that the relatively small sample of material needed for pesticide analysis is representative of the whole assignment? Also, once the representative sample has been taken, how should it be stored so that no changes in its composition occur until it is analysed? These problems and many more are dealt with in textbooks on sampling and standardisation, for example from the ACOL series, Woodget and Cooper's, *Samples and Standards*.[2]

Standardisation and Validation of Methods

If known quantities of standard reference materials (SRMs) – ideally isomers of target compounds – are added and thoroughly mixed into food samples at the outset (standard addition), subsequent methodology can be calibrated against

losses occurring during handling, to provide quantitative measurements of composition that can lead to the validation of the analytical procedure.

Recovery, Sensitivity and Limit of Detection

Measures of method performance, such as recovery, the limit of detection (LOD), and quantification (LOQ), are generally based on the use of standard addition and on the assumption that the additional standard material behaves like the natural substance in any physical and chemical treatments employed.

As far as the extraction process is concerned, the total recovery specified for the whole analysis includes the efficiency of the adsorbing medium, *etc.*, but, like all other parts of the assay, any losses that do occur are compensated for by the standard addition process. In practise, losses during extraction should be kept to a minimum, and for high sensitivity to be achieved in trace component analysis it is important to have as near a loss-free system as possible. With modern treated surfaces in separation columns and measuring instruments, and with the use of bonded stationary phases, there is less unwanted (irreversible) adsorption. Once receptor molecules of the target compounds have filled all the active absorption sites, any remaining molecules can proceed to the detector. The limit of detection is expressed as the threshold sensitivity of the detector to the remaining molecules, and is given a signal-to-noise ratio, *e.g.* 3 : 1. The LOQ is the lowest concentration of an analyte that can be determined with an acceptable precision and accuracy.

Precision, Accuracy, Reproducibility and Repeatability

Measures of reliability include the extraction stage, and errors of analysis need to be accounted for. Replication of the sampling and standardisation of the procedure is normal when quantitative measurements are being made, and a statistical evaluation of the reliability of this stage will be an integral part of the precision, accuracy, reproducibility, and repeatability of the whole analytical procedure. Most analytical methods provide this information and, therefore, it is assumed here that extraction is one of the processes, but not necessarily the limiting process, represented in the values arrived at for the whole method.

Certified Reference Material (CRM)

The importance of the provenance of the reference material used in the validation process is recognised, for example, by the European Union in the Fifth Framework Programme – the Measurement and Testing Programme. Two recent projects in the food analysis area are DIFFERENCE – Production of high quality CRMs for dioxins analysis in food and feed, and SPECIFIC MIGRATION – CRMs for control of migration testing of plastics for food packaging.[3]

Many CRMs for food analysis are standard matrices for interlaboratory comparisons of candidate methods. FAPAS has been instrumental in running

several trials[4] for the standardisation of data from analytical laboratories worldwide. Trials have been made on pesticides, toxins, veterinary drug residues, trace and nutritional elements, food colours, preservatives, sweeteners, alcohol congeners, fatty acids, nitrate, and proximate analyses.

The preparation of a laboratory reference material (LRM) of beef extract for heterocyclic amines (HA) determination was described.[5] Three levels of HA from 10 to 75 ng g^{-1} were added to the material, which was dehydrated, ground, sieved, homogenised, bottled and labelled for testing for suitability as a CRM in interlaboratory trials.

Measurement Uncertainty

Sample preparation is estimated to be the major stage of an analytical chromatographic procedure and the extraction process can make the major contribution to the total uncertainty of the assay. Therefore, the reader is referred to the *Eurochem/CITAC Guide*[6] and to the "Sample Preparation Perspectives" column[7] for the details on these and the seven hints for analysts:

1. Use adequate working techniques.
2. Use large volumes.
3. Minimise the number of working steps.
4. Make sample and reference measurements in a close time proximity and use the same instrument.
5. Use an internal standard.
6. Prepare an artificial matrix or use a certified matrix reference material.
7. Perform multiple analyses.

Remote Sampling

A modern approach to the automation of the sampling process is given in the "Process Column" article on extractive and remote sampling.[8] Four categories of sampling are given:

1. Non-contact sampling
2. Remote sampling
3. Extractive loop sampling
4. Grab sampling (remote off-line analyser)

Based around optical process spectroscopy, methods 1–3 are realising the objective of turning the whole analysis over to automation. Obviously, when the information required to quantify the analyte can be "extracted" remotely from the starting sample, extraction methods are redundant! There are several examples, *e.g.* using NIR spectroscopy where this is already well established. The authors discuss the state of the art.

In the meantime, it may be helpful to introduce the approach to sampling and sample handling as a prelude to dealing with the extraction processes.

3 Preparation for Extraction (Resumé of Extraction Aids)

Introduction

The raw food material may have to be subjected to some pre-treatment before an extraction can be performed effectively. Some food components are distributed throughout the whole cellular and intracellular structure. Superficial use of an extraction method would be inefficient, and ways of penetrating to the encapsulated or occluded analyte are categorised as pre-treatments or extraction aids. Some analytes are to be found only in specialised tissues that might be dissected from the whole and bulked (concentrated) to form the sample. In general, the preparation is to render the sample easier to extract. The main extraction aids are listed here and amplified in Chapter 2.

Change of Volume

Dilution aids processes where there is plenty of material, but where particulate matter might block filters or membranes. Conversely, trace amounts of analytes may be concentrated to increase the chance of detection.

Removal by dissection, often under the microscope, can enable parts of the food rich in a particular component to be bulked and used as a sample of smaller volume. This is a useful means of pre-concentration of the analyte. Dissection is also employed when interest is focused on only a part of the foodstuff, *e.g.* the seeds of a fruit or the intermuscular fat of a cut of meat.

Change of pH

The isoelectric point (pI) of an ionisable compound is the point at which the anion and cation contents are in equilibrium. A mixture of ionisable compounds, *e.g.* zwitterionic proteins, at a particular pH will often contain positively charged (below their pI) and negatively charged (above their pI) components. Separation can be effected directly by electrophoresis. In general, changing the pH of a food sample can facilitate the release of selected analytes. As an aid to extraction, it is often a prerequisite of membrane methods that the analyte is neutral and therefore a pH change will facilitate the transport of an analyte across the membrane.

Change of Structure (Cell Disruption)

Disintegrate and Homogenise

It may be necessary to break down the bulk structure so that the target components are accessible to the extractant. Very dry and hard foods (<5% moisture) are ground to a powder, *e.g.* in a mortar. Dry foods (<15% moisture) may be comminuted (disintegrated) in a blender, and wet foods liquidised to a slurry or

pulp. Blending or liquidising is often sufficient to render the sample homogeneous on the scale required for the extraction to be complete and reproducible from sample to sample. It is unlikely that the disintegration will release all the analyte, and over-zealous handling may cause decomposition, so a compromise has to be struck.

Biochemical Release

Enzyme hydrolysis (digestion) can be employed to degrade the cellular structure in order to release analytes from the matrix to provide a greater yield. Technology built up for vitamin analysis assumes several different biological states of the vitamin exist, and details the chemical classes from which the compound of interest is to be targeted for release. Mild acid and alkaline hydrolyses are used to release classes of chemical compounds that may be bound to structures or occluded in a chemical bond.

Chemical Release

There are occasions when the whole food has to be totally chemically digested to release the analyte. For the proximate analysis of protein, the food is digested in concentrated H_2SO_4 and the resultant nitrogen (representing the original protein) is converted into $(NH_4)_2SO_4$, which on distillation with NaOH releases NH_3 for steam distillation into a chemical trap of 0.1 M H_2SO_4 for subsequent titration against an indicator.

Microwave-assisted Extraction (MAE)

MAE is a sample preparation step in which internal vibrational energy is provided to the food matrix to release components into the liquid state or at least to render components accessible to extraction, *e.g.* solvent extraction.

Ultrasound-assisted Extraction (UAE)

Ultrasonics is another way of providing internal energy into the bulk of the material to interact with the structure and aid the extraction of components that otherwise would remain immobilised.

Change of State

Some soluble constituents can be treated with a chemical coagulating reagent, causing them to precipitate. In analytical terms, the larger the particle size precipitated, the easier will be the separation by filtration extraction. Small particles block filter beds and extend the separation time. Centrifugation is an alternative means of separation and works well with certain two-phase systems. The two layers are separated by decanting the supernatant phase, leaving the

compounds of interest more concentrated as either the coagulant or the supernatant. If necessary, the reaction product may be converted back into the original compound.

Additional heating, stirring or adding an electrolyte will be required if colloidal suspensions are involved and often the precipitation process will not be simple if coprecipitation occurs, taking down normally soluble material occluded to the precipitating particles. Factors affecting precipitation include, as well as particle size, solubility of the precipitate in the medium, temperature, reactant concentrations, the rate of mixing of the reactants, and the relative supersaturation and the balance between nucleation and particle growth (Skoog and West, 1982).[1]

Simply heating a sample can cause evaporation and, thus, extraction of volatilisable material from the matrix. Evaporation to dryness and condensation of the vapour phase separates solids and liquids and is, if taken to completion, an effective extraction process for stable analytes.

Dissolution will extract solubles for further treatment. Water, as a solvent, is very effective in many assays of solid foods. Heating or cooling for solid/liquid or liquid/solid to change the state is useful for analytes close to their transition point.

Change of Chemical Composition

It is often efficacious to add a chemical reaction into the protocol to avoid interference between the analyte and other co-extracted material at a later stage in the assay. There are many examples of derivatisation to increase the volatility of compounds for headspace (HS) analysis, or to change the retention time (RT) in chromatographic separation.

Flow Switching and Automation

The employment of instrumental methods under computer control is viewed as an extraction aid since processes like on-line flow switching (FS) may be used to effect extractions by diverting unwanted fractions away from the final separation stage. Other automated processes can also aid the extraction, such as continuous flow workstations with robotic arms that carry out several routine sample preparation steps and provide an extract for further study.

Flow Switching for Analyte Extraction in On-line Analyses

Flow switching, also called column switching, is a technique used in chromatography to change the direction of the mobile phase flow, *e.g.* to fill a sample loop with an aliquot from an external flow and then transfer it into the mobile phase flow to the separation column. When FS is used with a pre-column technique, sample loading onto the pre-column can take place with the eluent venting to waste until, *e.g.*, unwanted components with a low affinity for the sorbent have been extracted to waste. Then, by switching the flow to a mobile

phase with greater solubility for the COI, the analytes can be transferred to the analytical column for separation in an automated process. It is possible to effect front- middle- and end-cutting of the adsorbed fraction in this way. These processes are seen as assisting in the extraction.

Automated Preparative-scale GC Injections and Fraction Collection

The use of carousels, automatic injection systems, and fraction collectors provide mechanical assistance in the preparation of samples for separation and fractionation–extraction. Dilution or chemical reaction, *e.g.* derivatisation, may be performed robotically on the sample for analysis and the extracted fractions subjected to further separation.

Miniaturisation

The introduction of benchtop mass spectrometers to replace the floor-standing instruments of the 1960s and 1970s started the move towards small footprint modules for complex, multiple compound analysis. The combination of GC with MS brought further reductions in the overall size of "benchtop" instrumentation. As the number of assays, and the number of analytical steps that are coupled together increases, the need for further miniaturisation continues. Nanotechnology on the molecular scale may be a future development in analytical methodology, but, for now, microchip instrumentation is moving apace, and examples of combined sample preparation, separation and detection on a chip using capillary electrophoresis (CE) technology are given.

4 General Approach to the Extraction of Analytes

Phase Separation

Many foods and food products are natural polyphasic systems and simple phase separation methods may remove unwanted fractions of the matrix. Alternatively, maceration can be used to produce a slurry that may be physically separated into solid and liquid fractions. The common use of an organic solvent to remove certain soluble components from the aqueous food matrix depends on the partition ratio (k) of the analyte between the two phases. If an analyte has a significantly different ratio from that of other constituents, then an extraction is practical. The greater the difference the more likely it is that a single step extraction will produce a clean enough sample for the separation stage. Components of a mixture that have only small differences in k require multiple extractions by the same, or different, methods.

Filter Bed

The simplest form of phase separation is filtration. If there has been a separation of phases so that some of the sample is in the liquid and some in the solid state

then providing that the particles of the solids are greater than the pore size of the filtration medium they will be retained on the filter bed. Filters are defined by their particle retention size and speed of filtration, and a wide range of papers from 2–30 μm, glass fibres from 0.5–2.5 μm and frits of approximately 70 μm, and membranes (nylon, PVDF, PTFE, *etc.* with pore sizes around 0.2–1.0 μm) with speeds between 20 and 2500 s per 100 ml are manufactured to accommodate the extraction. Losses will occur and either standardisation or exhaustive washing is required to retain a quantitative recovery.

Separating Funnel

The distribution of analytes with different partition constants between two immiscible liquid phases enables a physical separation. If, after a time for equilibration, the amount of the COI in one phase greatly exceeds the amount in the other then a single-stage extraction in a separating funnel might be sufficient to separate it from interferents. This applies especially to the mixing together of a liquid food and a solvent in a separating funnel. Careful choice of solvent can extract different chemical classes quickly and efficiently.

Filter Funnel

If solid has formed in a liquid food, or if a comminuted food matrix contains sufficient liquid phase, the use of a suitable porosity filter paper will extract the solid, and purify the liquid food, for further study. Filtration processes are involved in most of the sample preparation for extraction protocols encountered in food analysis. Compensation against analyte loss is necessary.

Büchner Funnel

A range of sizes and porosities of fixed glass frits, and the ability to add a medium such as Celite as a filtration aid, makes the Büchner funnel invaluable in food analysis.

Centrifuge

The soluble and insoluble components of a food matrix can be separated by centrifugation, after which an extraction can be made by decanting the supernatant liquor. Components of the sample may be deliberately precipitated and separated by filtration or centrifugation from the solubles in the supernatant liquor. Many food assays contain a centrifugation step, and an interesting application is in the preparation of bacterial cultures for polymerase chain reaction (PCR) analysis:

Buoyant Density Centrifugation (BDC). In food microbiology, BDC is used to prepare samples for PCR analysis. The density gradient was externally

calibrated using density marker beads (Pharmacia Biotech, Uppsala, Sweden) and the buoyant densities of bacterial strains and food homogenates were determined by centrifugation in a continuous density gradient. 1.7 ml 50% stock isotonic solution [100% stock isotonic solution: 100 ml BactXtractor™ (QRAB, Uppsala, Sweden), 850 ml NaCl, and 100 mg peptone] was placed in a 2.2 ml test tube and 0.5 ml of analyte layered on top. Alongside the analytical tube, the calibration tube was filled with 0.5 ml peptone water and 5 µl each of 7 different density marker beads placed on the gradient medium (50% stock isotonic solution) surface in place of the analyte. Tubes were centrifuged at 16200*g* for 7 min and buoyant densities determined against a calibration curve.[9] The method was optimised and, after centrifugation, the supernatant was removed, leaving the bacteria at the bottom of the tube. The tube was filled with phosphate buffer saline and the bacteria pelletised at 9500*g* for 5 min, the upper layer again removed and 75 µl volume containing the bacteria was taken for PCR analysis. During development of this method, processed brawn, raw beef and raw minced pork were used as samples. (Summarised from ref. 9 with permission from Elsevier)

Decanting

When centrifugation, precipitation, simple settling or sedimentation has separated the liquid and solid phases, the liquid phase can be decanted to extract the soluble components. When distribution ratios are less distinct, multiple extractions, multistage separations or more complex procedures such as countercurrent distribution are necessary.

Distinction between Separation and Extraction

Continuous partitioning from a mobile phase while it is passing over or through a stationary phase is a chromatographic separation in analytical parlance. Now that solid-phase extraction (SPE) methods are important in the preparation of samples for subsequent chromatographic separation, it is convenient to consider pre-separation methods as extraction methods and separation methods as those operated with on-line detection of the components (fractions) of the sample mixture. This is only a guide, since it would be feasible to couple a detector to some extraction methods, but the prime objective of an extraction is to simplify, or purify, a sample for further chromatographic and spectroscopic examination. The distinction is blurred by preparative-scale chromatography performed to concentrate and separate components of a complex mixture, the result being a number of distinct fractions for further study.

Most extraction methods employ some form of partitioning such that a component or components of the food are removed from the matrix. Processes such as distillation, solvent extraction, SPE and countercurrent distribution are partitioning processes. Normally, components that are extracted can also be concentrated, either by selective adsorption and extraction in a small volume of a different solvent or by solvent evaporation where the analytes are significantly less volatile than the solvent.

Consider the Resolution of the Total Assay

The objective for the extraction step is to remove as much of the bulk matrix as is necessary for the analytes to be recognised and/or quantified unambiguously in the subsequent steps in the analysis. At one time, this was a rigorous requirement, but as the separation and, particularly, the detection stage increased in resolving power there was less need for absolute purification at the extraction stage, and therefore it was necessary to evaluate the whole procedure in order to optimise the performance/analytical effort factor. Conversely, as the resolving power of sample preparation methods improves, less resolution is necessary at the later stages of the analysis, again requiring optimisation to avoid overkill. In designing a screening method for carbamate and organophosphate pesticides in food matrices, the use of an electrochemical bioassay meant that a lyophilised solvent extract of homogenised food could be used directly, whereas for GC and HPLC analysis an additional C_{18} SPE and a salted out organic extract was required.[10]

The use of ECD-GC and NPD-GC for pesticide analysis elicited the comment that a simple UAE with acetone–DCM over anhydrous NaCl was sufficient and no further clean-up was necessary (Navarro *et al.*, 2000, Chapter 2, ref. 16).

High-resolution Detection

High-resolution mass spectrometry detection can often provide additional resolving power for would-be interferents at the end of the assay. Small differences in the fractional mass of ions detected may be specific to the target compound and not to isobars (ions of the same nominal mass but of different atomic composition). In addition, using MS^n techniques provides "dry" ion separation analogous to "wet" chromatographic separations as on-line detection procedures. Therefore, it may be unnecessary and inefficient to spend time finessing the removal of potential interfering substances at the extraction stage, making it more important to design the assay as a whole. Optimisation of the corporate parts of an assay to obtain the most efficient use of resources can be a difficult and time-consuming operation. Consequently, where researchers are known to have gone to the effort to report their experiences at optimisation, they have been referenced here. It is a sinecure that time spent in successful optimisation, leading to a decrease in analysis time, is recovered handsomely in the repetitive routine assay.

Special Case of Labile Samples

When the sample is sensitive to light or heat, special extraction conditions have to be used. It is mandatory to work in the dark at reduced temperatures when handling, *e.g.*, carotenoid samples.

Special care is needed when analysing cooked foods containing labile compounds. Many of the nutrient, pigment, and vitamin values change during the cooking processes and, therefore, the state of the cooked food, or the details of

the cooking process, have to be added to the description of the analytical protocol. Additional problems occur, especially for nutritional assays, when oil is added during the cooking process.

Where carotenoids are concerned, in fresh fruits and vegetables, their biosynthesis continues during storage and can cause errors when raw and cooked foods are compared. For these and other precautions and methods of calculation for labile components, the paper by de Sá and Rodriguez-Amaya is recommended reading.[11] For carotenoid extraction from cooked foods, they preferred to disintegrate the sample with cold acetone in a mortar rather than in an electric blender, and for raw foods an acetone pre-treatment in an ultrasonic bath for 20 min was used.

Other stages in the extraction of carotenoids included processes listed here and explained in the appropriate chapters later:

1. Stir-fried material cooled in a freezer for 2 h to solidify the oil.
2. Filtered in freezer using cold glass-sintered funnel.
3. Partitioned with 10% ethyl ether in petroleum ether.
4. Saponified with equal volume of 10% KOH in MeOH, added to petroleum ether extract containing 0.1% BHT (mixed at room temperature in the dark).
5. Washed.
6. Concentrated in rotary evaporator.
7. Dried under N.
8. Redissolved in filtered acetone.

Classification of Plant Crops for Extraction

The *Codex Alimentarius Commission* has classified plant crops into 24 botanical types. This may be a useful record for the food analyst because it may help to categorise extraction methods by commodity.[12] This was addressed and 6 groups of plants have been recognised and classified.[13] Briefly, the classes are:

1. Root and bulb vegetables.
2. Low chlorophyll and oil content fruits and vegetables.
3. High chlorophyll plants and crops (excluding high oil content commodities).
4. Dried fruits of high sugar content.
5. Dried crops of low oil content (that can be powdered).
6. High oil content crops.

Classification of Foods for Pesticide Analysis

In the area of pesticide analysis, food materials have been classified according to the solvent system used for their extraction.[14] Groups I and II, vegetables, fresh fruits, whole milk, green cheese, eggs and meat are extracted in acetone, while groups III and IV, cheese, dried legumes, wheat meal, pasta, rice and bread, require acetone–water. For a more comprehensive review of this

classification system for pesticide analysis, consult Tekel' and Hatrík (1996) (Chapter 8, ref. 77).

5 Resumé of Extraction Methods

Introduction

Within the general principle of partition, four physical processes have been recognised in the extraction of analytes from foods: solvation, distillation, adsorption, and diffusion. All other associated processes: percolation, filtration, precipitation, microwave radiation, enzyme hydrolysis, *etc.*, which assist in the release or removal of components from the bulk material are considered to be extraction aids and are dealt with in Chapter 2.

Partition (Chapter 3)

Introduction

Partition is the fundamental process whereby a chemical compound in a food matrix transfers to an extractant. Partition constants quantify the efficacy of the extraction.

Partition–Extraction

If two compounds are soluble in two immiscible solvents to different extents, by mixing the two solvents containing the compounds, until a dynamic equilibrium is created, the compounds will be distributed between the solvents according to their partition or distribution constants. In practice, solvent pairs can be chosen so that, at equilibrium, the solutes (analytes) are substantially separated when the solvents are separated. This method of extraction is common in the food flavour industry and has general applications in food analysis.

Gas/Liquid, Liquid/Liquid, Solid/Liquid Partition

GLP, LLP and SLP are terms defining the states of matter involved in the distribution. The time taken to establish equilibrium between the two states varies considerably with the composition of the binary system.

Microdiffusion Extraction (MDE). Volatile components evaporate into the headspace around foods approximately according to their air/water partition constants. The temperature may be raised to increase the rate of (a) the formation of volatiles from involatile precursors and (b) the rate of their vaporisation from the liquid state. Volatiles are then concentrated by condensation at a small volume external site or trapping in-line chemically for subsequent controlled desorption. In a way, the natural evaporation process is a microdistillation, or a microdiffusion, of molecules that can enter the gas phase. If time is not important, microdiffusion as a method of extraction is effective and cheap.

Solvation (Chapter 4)

Solid/Liquid Extraction. Solvent extraction is a particular case of partition. If an immiscible solvent is added to a comminuted food sample and the sample shaken, first the particles swell by sorption and capillarity and then diffusion from the solid into the solvent occurs for any food components soluble in the solvent, and at equilibrium they will be present in the solvent in concentrations proportional to their partition constants. In favourable cases, most of the component can be extracted into the solvent. In special cases, hydrolysis will help to increase the release of otherwise unavailable components. The extraction process is governed by:

1. Nature of the solvent.
2. pH of the medium.
3. Particle dimensions.
4. Temperature.
5. Volume of solvent.
6. Number of extraction steps (3×20 ml rather than 1×60 ml).

Matrix Solid-phase Dispersion (MSPD). MSPD is a new approach to the optimisation of the extraction and clean up, *e.g.* for multi-residue methods (MRMs) in pesticide analysis. The finely dispersed food matrix is placed in a column and mixed with a solid-phase adsorbent such as Florisil, and the target compounds eluted selectively from the dispersed sample with organic solvents.

Sub-critical Fluid Extraction. Solvents raised to temperatures and/or pressures near to the critical region exhibit properties conducive to efficient solute extraction. Several methods are available for food analysis:

1. Pressurised Liquid Extraction (PLE). The efficiency of the extraction increases as the temperature and/or pressure of the liquid extractant approaches the supercritical region.
2. Subcritical water extraction (SWE). Hot-water extracted analytes mix under pressure with an organic solvent. The mixture is then cooled rapidly and the water enters a polar, sub-critical state and partitions with the organic solvent.

A closely related method is,

3. Pressurised Hot-water Extraction (PHWE). The dielectric constant of water decreases as the temperature increases, and non-polar analytes dissolve easier in low dielectric constant solvents. This method is finding uses for contaminant analyses.

Supercritical Fluid Extraction (SFE). A supercritical fluid is similar in properties to a dense gas. The use of non-toxic CO_2 as the extractant has been in use for many years on commercial and analytical scales.

Distillation (Chapter 5)

If food constituents can be volatilised without decomposition, then they can be concentrated by condensation into an extract. This extraction is effected in practice through the process of distillation. Historically, volatilisation, distillation and fractionation are associated with the production of food essences. More recently, the analysis of food flavour compounds has used these processes, and the culture of chromatographic separation is founded on these principles. The Theoretical Plate (TP) is a concept of separation efficiency in which a TP is considered to be a volume in a fractionating column, or a chromatographic column, large enough in which to achieve equilibrium between the mobile and the stationary phases. At equilibrium, solutes will be distributed between the two phases according to their partition constants. This volume reduces as the efficiency of the column increases. A reflux fractionation process can be used to separate substances that have sufficiently different boiling points and therefore condense at different places (heights) in the reflux condenser. The width of the band occupied by the condensed material constitutes a plate, and the more efficient the column the narrower the plate. Hence the concept of the height equivalent of a theoretical plate, (HETP, or simply *H*).

Steam Distillation (StD). Since many foods contain water, and many more are prepared for eating by cooking in water, steam distillation is a very important process in food science. In food analysis, it serves to extract volatile materials to an external collector for further analysis.

Organic Solvent Distillation–Extraction. Mainly used for the extraction of water from food samples, but, in principle, a higher boiling non-polar solvent volatilises a lower boiling polar solvent, carrying it over during a distillation. If the polar solvent is the more dense then it can be collected below the non-polar distilling solvent and collected into a burette for quantitative measurement. [see Dean and Stark distillation].

Simultaneous Steam and Organic Solvent Distillation–Extraction (SDE). The Likens–Nickerson steam distillation/solvent distillation–extraction method has found many applications in flavour analysis. A steam distillate of the volatile material from a food sample co-condenses with the vapour of an organic solvent. The mixed condensates separate, with the denser material below, and return to their respective boiling flasks. Thus, after an hour or two the condensable, organic soluble volatiles are transferred continuously from the water vapour to the organic vapour and concentrated in the organic solvent distillation flask.

Countercurrent Distribution. Continuous agitation of a binary phase system (two immiscible solvents), such that one solvent moves in a direction opposed to the flow of the other (countercurrent), will enable equilibria to be established

frequently between the solvents, allowing solutes to be distributed effectively in concentrations proportional to their partition constants.

Sweep Co-distillation (SCoD). An inert gas at high temperature is used to sweep out volatilisable material for downstream condensation from a sample mixed with a solid packing in a glass tube.

Adsorption (Chapter 6)

A very useful physical phenomenon is the adsorption of molecules to solid (or liquid) immobilised particles. The reversibility of the adsorption defines the type of extraction or separation that can be effected. A one-off irreversible extraction can remove unwanted material so that a less strongly adsorbed analyte can be eluted for further treatment. However, if the analyte and the interferents have different affinities for the adsorbent then reversibly adsorbed compounds can be eluted sequentially in an extraction protocol. The chromatographic technique is the vehicle used in modern analytical chemistry to achieve multiple equilibration steps – and hence separations – according to the distribution constants of the solutes. In solid/liquid chromatography, the distribution constant (K) is the ratio of the concentration of analyte in the stationary phase (C_s) to that in the mobile phase (C_m).

$$K = \frac{C_s}{C_m} \tag{1.1}$$

The value of K for analytes determines their order of elution. Low K analytes elute earlier than those with higher values. In terms of an extraction protocol, a large difference between the K values of compounds in a mixture may be used to design a separation/extraction strategy.

Solid-phase Extraction (SPE). The rules of liquid chromatography apply to SPE. A tube filled with an adsorbent powder is wetted with a solvent and then a sample in the same solvent is applied to the top of the column. Either the compound of interest washes through with the solvent and the contaminants are retained on the column or the COI is retained on the solid phase while some contaminants elute with the solvent. Changing the nature of the solvent may be used to elute selectively other contaminants adsorbed by the solid phase. Finally, the COI may be concentrated by being eluted in a small volume of a suitable solvent. There are six general categories or modes of extraction.

1. Adsorption
2. Bonded phase partition
3. Normal phase
4. Reversed phase
5. Ion-pairing
6. Ion-exchange

Liquid foods may be applied directly to the adsorbent phase and washed through to leave the analytes adsorbed for subsequent elution (Holland *et al.* reference 38 in Chapter 6).

Solid-phase Microextraction (SPME). Utilises the same principles as SPE, but in practice is a microprobe bearing a support-coated liquid phase on a fused silica fibre in a stainless steel sheath (needle), allowing the retracted microprobe to be injected into the heated inlet zone of a gas chromatograph. Fibres can be immersed in some liquids and suspended in most headspace environments above liquid and solid samples. Volatiles and semi-volatile substances are adsorbed on the bonded stationary phase and desorbed by heating, normally in the GC inlet, or eluted with a solvent, normally in the LC inlet, where they will be separated for further analysis. SPME is a solventless extraction technique providing on-line transfer of the extract to the separation stage.

Multidimensional Solid-phase Chromatography in the Extraction Mode. Most solid-phase chromatography techniques can act as extraction systems:

1. When on-line dual chromatography is in use, the first separator acts as an extractor for the second stage process.
2. As off-line separators when combined with fraction collection of the eluent.
3. When a guard column is used to protect the main analytical column. The guard column extracts unwanted components of the sample or the mobile phase.

Chromatographers will be familiar with "heartcutting" of a fraction from one column and transfer to a second column, usually with a different polarity phase, to take full advantage of the two-dimensional separation. For many years the 2D approach was accomplished through stainless steel switching valves, but problems of dead volume and extra path length limited the resolution of the on-line system. Off-line methods were slow and liable to sample degradation during the transfer. The valveless switch was introduced in 1968 that used a pressurised auxiliary gas flow to balance and, when required, re-direct the carrier-gas flow.[15] Recent applications use this principle and apply modern pressure control and sensing systems. The outcome is the automated transfer of selected fractions from column 1 on to column 2 for final separation and detection. Together with modern software-controlled decision making, 2D analytical methods are feasible for automated assays.

Special Case of Preparative-scale Chromatography. Large-scale chromatography has always been used in conjunction with fraction collectors to separate components for further study. With modern detectors having higher sensitivity, smaller amounts of purified material that are sufficient for subsequent high sensitivity separation stages can be made available.

High Concentration Capacity Microextractions. Methods such as stir-bar sorptive extraction (S-BSE) increase the capacity of adsorbent over that used in SPME to extract analytes from aqueous food matrices. Like SPME they are solventless and capable of on-line operation. However, the increased amount of extract requires longer to desorb than is possible during thermal desorption in the inlet to a GC, if high resolution is to be maintained. Therefore, purpose-built desorption units are commercially available. Desorbed material is cold trapped and re-concentrated for injection into the chromatographic system.

Diffusion (Chapter 7)

Introduction. The diffusion of molecules or ions from a point of high concentration to one of lower concentration governs the movement of analytes across membranes in processes like dialysis, permeation, *etc.* Fick's law governing the mass transfer states (Equation 1.2):

$$M_s = -DA\frac{dn}{dx} \tag{1.2}$$

where, M_s is the mass of analyte carried across an area A of a surface normal to the direction of diffusion per second, and n is the analyte concentration at an arbitrary point x. Classically, membrane separations were viewed as diffusion processes, and have been segregated here under this heading.

However, in practice, a complex physical relationship exists between aqueous food matrices and membranes that permit the passage of certain components through their structure. Membranes of porous impregnated or non-porous structures are used to separate two liquid phases, and therefore adsorption and solvation processes will also affect the mass transfer. In general, the molecular size determines the permeation selectivity of the membrane.

Permeation and percolation processes involving solution and adsorption can be used to great advantage in the extraction of chosen components. Dialysis, which involves both molecular size and ionic charge, can be valuable when coupled to ion separation methods such as CE[16] and capillary ion chromatography (CIC).

Microporous Membrane Liquid/Liquid Extraction (MMLLE). If a thin hydrophobic microporous membrane separates two immiscible liquid phases, *e.g.* one aqueous and one organic phase, solutes can undergo mass transfer.

Membrane-assisted Solvent Extraction (MemASE). The technique uses a sample vial into which a membrane bag attached to a steel funnel insert is fitted under a septum-carrying metallic crimp cap such that a few microlitres of solvent may be placed in the membrane bag and the bag immersed in a volume (*e.g.* 15 ml) of aqueous sample. Diffusion of hydrophobic analytes into the organic solvent is sampled by hypodermic syringe for large volume injection (LVI) into a GC.

Sorbent Impregnated Membranes. Chemically modified membrane surfaces are "tailored" to suit the extraction. A specific chemical group can be impregnated to provide a high degree of specificity to the transport across the membrane. Ion exchange membranes have positively or negatively charged groups covalently attached to the polymer.

Supported Liquid Membrane Extraction (SLME). SLME is a three-phase extraction technique where two aqueous phases are separated by a thin, porous hydrophobic membrane carrying an organic liquid by capillary action.

Pervaporation. The donor liquid flow may be a fruit juice, and pervaporation involves the diffusion of gaseous substances dissolved in the aqueous phase across a hydrophobic membrane into the acceptor stream.

Dialysis. Dialysis separates sample components depending largely on their size relative to the pore size of the membrane, but other factors relating to the donor and acceptor liquids and physical properties of the analytes will need to be considered. In practice, various modes of operation have been described.

Osmosis. Osmosis is the passage of analyte molecules from a less concentrated to a more concentrated solution through a semipermeable membrane until both solutions are at the same concentration. Osmotic pressure is the pressure necessary to prevent the movement of molecules across a semipermeable membrane to establish equal concentrations on both sides. Typically, water molecules are extracted from liquid foods into concentrated brine solution.

Filtration. The most common method of liquid/solid phase separation effecting an extraction of soluble from insoluble constituents of a food matrix is filtration. The matrix may contain a natural binary system or the food can be comminuted to form two phases. In chemical analysis there are many materials that are permeable to liquids that act as barriers to solids. In practice the porosity of the barrier defines the completeness of the phase separation, and suspended particulate material may inadvertently pass through high porosity filters. Conversely, in practice, low porosity filters block too quickly to be effective. Modern membranes have been developed to produce a working compromise for many analytical purposes.

6 Proximate Analysis of the Major Food Components

Introduction

Proximate analysis of a food sample determines the total protein, fat, carbohydrate, ash, and moisture reported as the percentage composition of the product. There are food composition tables that contain proximate analyses for a large

number of established foods, and as new food items are added to our shopping baskets their proximate compositions are added to the database, periodically, in supplements.

Data contained in food composition tables and the analytical methods used to produce these data are continually under review[17] and regulatory bodies publish modifications as they reach a level of general acceptance and reliability. The quality of the assays and the definition of the composition (*i.e.* which components are included in the measurement) vary. The diverse range of analytical methods used introduces small differences among the compositional values that require the source to be identified and RSDs to be reported with the data. Extraction methods used in proximate analysis of the major constituents of foods and food products are outlined here and developments are discussed in subsequent chapters. Preparation methods used in conjunction with proximate analyses are described in Chapter 2.

The subject is approached from the classical viewpoint and updated from the recent literature.

Total Protein

The total protein content of a food sample is estimated as total nitrogen (*e.g.* the Kjeldahl method) after digestion, salt neutralisation and titration of the ammonia released against standard acid. A conversion factor is applied to calculate the total protein. Some functional groups, $-NO_2$ and $-N{=}N-$, do not react and need further treatment if their omission will make a significant difference. Even the classical Dumas' method gives different figures for some foods compared to the Kjeldahl method. There are now many modifications that have been developed to cope with a wider range of food matrices.

Total Carbohydrate

There are disputes about what should be included in the calculation of carbohydrate content. The chief difference lies in the reporting of "total" carbohydrates, made up of monosaccharides (sugars) and polysaccharides (starch and cellulose, including soluble and insoluble fibre). Should fibre be included or not? Some analysts report fibre separately and others include it along with the available sugars and starches to give total carbohydrate. Current qualitative and quantitative interest in fibre for nutritional marketing and food labelling requires a separate figure to be available anyway.

A further anomaly lies in the practice of reporting total carbohydrate as the difference, after summing the quantities of the other components. The analytical implications of these uncertainties are discussed in relation to extraction methods.

Total Lipid (Fat)

Total lipid (fat) content may be calculated simply as the material extracted into diethyl ether. However, there are concerns over the availability of the many

chemically different forms of fat and at least a digestion of the protein and carbohydrate would ensure the efficient release of fat from the tissue. Modern solvent-extraction methods are employed to improve the reproducibility, but there are still discussions about the nature of the lipids extracted under the different conditions. In addition, food labelling requirements entail further separation into saturated, polyunsaturated and monounsaturated fractions. Furthermore, recently, the omega 3 fatty acids have been reported separately owing to their importance in healthy foods.

Moisture Content and Total Solids (Ash)

Moisture is measured as a mass difference after dehydration, and total solids or ash is recorded as the material remaining after the removal of all vaporisable material by high temperature combustion in a furnace (*e.g.* at 500 ºC). Moisture may also be determined by chemical reaction. Again, the debate continues over the definition of extractable (or reactable) water.

Water Content – Direct Methods

Azeotropic Solvent Distillation

Water is the major component of most fresh foods and, therefore, the determination of moisture content defines the dry matter composition, a more quantitative starting point in the measurement of the nutritional value of the food. The Dean and Stark azeotropic solvent distillation–extraction method has been in use for many years (Chapter 5). The water is co-distilled by the solvent, *e.g.* toluene, and condensed into a side arm for volumetric estimation. The use of large amounts of organic solvents and the long time taken to reach a constant reading of the water distilled over has motivated the development of alternative methods.

Mass Difference

Evaporative Heating (Dehydration). A simple method of measuring moisture content is to heat the food to dryness by evaporating the water into the atmosphere (oven method) – a direct form of total volatile extraction – and measure the loss in mass. The temperature of evaporation has to be carefully chosen so that thermal decomposition of labile substances is minimised to avoid adding to the volatile loss, assumed to be water. The long time taken to reach a constant residual weight has stimulated the search for other methods.

Carter-Simon Moisture Meter. The Carter–Simon moisture meter is operated at 150 ºC. Approximately 7 g finely ground sample are heated in an oven for 20 min, followed by cooling in a desiccator for 10 min. The loss in weight is "calibrated".

Rapid Radiant Heating. Infrared and microwave drying methods are faster and more reliable since modern automated instruments remove inconsistency.

Desiccation

Another simple method is to place the wet sample in a desiccator with a strong desiccant, such as P_2O_5, and weigh the sample at intervals until equilibrium is reached.

Chemical Reaction and Volumetric Titration

Karl Fischer Titration. The classical Karl Fischer titration method was developed to answer some of the above criticisms.[18] MeOH reacts with SO_2 in the presence of a base to give the methyl sulphonic acid anion, which is then oxidised by I_2 if free water is present (Equation 1.3).

$$3B + MeOH + I_2 + SO_2 + H_2O = 3BH^+ + MeOSO_3^- + 2I^- \quad (1.3)$$

The amount of iodine consumed is measured by coulometry or volumetric titration and related quantitatively to the amount of free water present. The method is totally dependent upon the comminution of the sample matrix and modern high-speed mixers are used to disintegrate the cellular structure. Some help can be gained from solvent mixtures to dissolve certain foods. A whole book of recipes has become available to cope with multi-component foods containing starch, fat, and protein in different proportions and in different structured forms.

Modern development has been to find less toxic reagents[19] and to automate the sample preparation and titration. The Mettler-Toledo DL 38 Karl Fischer Titrator is an example.[20]

Combinations of Direct Methods

Evaporation and Titration. If the water vapour released during heating is directed through a Karl Fischer titration cell the moisture content may be calculated from the titre.

Evaporation, Hydration and Electrolysis. If the water vapour released during evaporation is passed through a tube of P_2O_5, the phosphoric acid hydration product may be electrolysed and the H_2 and O_2 measured.

Water Content – Indirect Methods

Rapid and Remote Sensors

NMR Spectroscopy. Free water (the H nucleus) is detected and the relaxation time is related to the physical environment of the nuclei. Again, a calibration is necessary for every food matrix.

NIR Spectroscopy. The use of NIR/IR spectroscopic methods introduces the additional capability to measure other components too. The "reading" has to be calibrated for every food matrix. FT-IR and chemometrics enable remote sensors to measure water and other parameters in "at-line" mode in food processing plants.

Microwave Spectroscopy. The wavelength shift and amplitude attenuation depend upon the water content. Averaged multiple wavelength measurements improve the precision.

Total Solid

Evaporative Methods

The residue after the extractable water has been evaporated is called the total solids. It is an essential part of the proximate analysis. Traditionally, oven evaporation methods have been used. By way of example, direct forced-air oven-drying methods were described in a very detailed and well-controlled collaborative study using AOAC method 990.20 for milk and were published in 1989. After collaborative studies over several years a report was made on the performance of the trials.[21] Repeatability and reproducibility improved over time.

In-line Sensors

In the food industry, in-line sensor methods have been developed for the measurement of total solids/moisture content, *e.g.* in continuous fruit juice processing.[22] NIR, guided microwaves and the Maselli refractometer were evaluated against the Abbe refractometer for the continuous processing of apple, grape, pear, apple-cherry and apple-banana juices. The automated methods gave good results over prolonged use without problems of deposit build up.

Total Lipid

Acid Digestion

A simple method of measuring the total fat content of food is to digest a sample in concentrated H_2SO_4 and measure the remaining lipid layer in a graduated tube. The fat content of tree nuts, peanuts, sunflower seeds, avocado and olives was determined using this method,[23] which is conveniently performed using the Gerber Tube method developed for milk lipids (see Solvent Extraction Methods for Lipids in Milk and Cream below). The results agreed with the Soxhlet extraction method.

SFE and Enzyme Transesterification

The rapid measurement of total nutritional fat content of meat used SFE at 12.16 MPa and 50 °C. The extract was transesterified with MeOH and catalysed

by an immobilised lipase. SFC was used to monitor the conversion of the triglycerides into FAMEs and the FAs were analysed by GC. Total fat, saturated fat and monounsaturated fat contents were calculated from the GC data.[24]

Solvent Extraction Methods for Lipids in Milk and Cream

Regularly used extraction methods for lipid analyses of milk are:

1. Soxhlet extraction (hot method)
2. Soxhlet extraction (standard method)
3. Bligh and Dyer extraction
4. Modified Bligh and Dyer method
5. Roese–Gottlieb extraction
6. Gerber method
7. Fleet and Linzell centrifugation
8. Creamatocrit method
9. Babcock method (milk)
10. Babcock method (cream)
11. Modified cream Babcock method
12. Mojonnier method (cream)
13. Modified Mojonnier method

Total lipid determination was the subject of an article comparing five solvent extraction methods, two solvent distillation and three liquid/liquid extraction (LLE) methods of analysis (1–5) above (Table 1.1) for five commodities.[25] These methods are all discussed under the appropriate extraction principle.

The Gerber digestion extraction (method 6) for measuring fat in raw and processed milks was the subject of a collaborative study in 2001.[26] The method is simple and rapid, but the volume of sample used seems to vary. A collaborative study was conducted to determine whether test portions by weight or by volume (11.13 g or 10.77 ml) were better. Eleven laboratories participated in the evaluation of aliquot addition by weight and ten laboratories participated in the evaluation of aliquot addition by volume. The Mojonnier ether extraction (MEE) method was used as the reference method. The fat content of milk samples ranged from 0.96 to 5.48%.

The Fleet and Linzell method (7)[27] simply separates (extracts) the fat from the aqueous layer of the milk by centrifugation, and the Creamatocrit method (8)[28] uses a capillary tube as a microcentrifuge tube, filled with milk, sealed, and spun at 15000 rpm for 5 min. The fatty phase length was measured as a % of the tube length occupied by the total sample. The method was compared to the Gerber method ($R^2 = 0.968$), and was preferred because of the danger associated with the concentrated acid digestion involved in the Gerber method.

The complex nature of the total lipid composition of foods, from non-polar glycerides to polar phospholipids, means that solvent extraction – which is the most common method – has to be effective across a range of polarities. This is made more difficult because lipids bind to proteins (lipoproteins) and sugars

Table 1.1 *Five total lipid determination methods compared for five commodities*
(Reprinted with modification from the *Journal of Food Composition and Analysis*, vol. 14, P. Manirakiza, A. Covaci and P. Schepens, "Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh and Dyer, and Modified Bligh and Dyer Extraction Methods", pp. 93–100, © 2001, with permission from Elsevier)

Method	*Measurement*	*Chocolate powder*	*Milk powder*	*Liquid milk*	*Margarine*	*Eggs*
	Producers content (mg g^{-1})	32	5	36	362	98
Bligh & Dyer	Mean	33	3	27	317	95.5
	RSD (%)	2.0	2.2	3.3	0.4	2.6
Modified	Mean	33.4	4.9	32	282	91.5
Bligh & Dyer	RSD (%)	2.3	0.4	0.5	0.4	1.1
Roese–Gottlieb	Mean	25.6	4.1	11.2	337	41
	RSD (%)	2.5	1.1	1.4	0.7	2.4
Standard	Mean	31	4.1	14.5	349	11(30[b])
Soxhlet[a]	RSD (%)	1.4	2.7	1.5	0.5	1.7(1.5)
Hot Soxhlet[b]	Mean	34.7	4.8	17.4	362	12.5(39[b])
	RSD (%)	2.1	1.7	2.3	0.5	1.2(2.0)

[a] Acetone–hexane (1:4). [b] DCM–hexane (1:4).

(glycolipids) on cell membranes, requiring a particularly polar solvent to remove them. Over the years several extraction methods have been devised, but the above methods represent the main usage in food analysis.

A collaborative study (11 laboratories) was carried out in 1988[29] using the Babcock method with the MEE method as standard for measuring the fat content of milk. Ten laboratories used the modified Mojonnier ether extraction (ModMEE) method and 10 used the Babcock extraction (BE) method. The ModMEE method gave consistently better within- and between-laboratory agreement. The overall mean test value for the BE method was significantly higher (0.021% fat) than that for ether extraction. The modifications of the AOAC Babcock method and the ModMEE method have been approved interim official First Action by the AOAC.

A collaborative study[30] in 1996 was set up to rationalise the analytical methods for the fat content of cream. The ModMEE method for the fat content of cream was developed along the lines of the method for milk (AOAC, OM 989.05). The cream Babcock method (AOAC, OM 920.111B-C) was modified to harmonise with the milk Babcock method (AOAC, OM 989.04) and also to clarify procedural details. Ten laboratories tested 9 pairs of blind duplicate heat-treated cream samples with a fat range of 30–45% using both methods. The ModMEE and Babcock methods for fat in cream have been adopted by the

AOAC. The new Babcock method replaced the AOAC Official Method 920.111B-C.

Recently, the same team published further collaborative studies on a modified Babcock method aligned with the MEE method[31] (Appendix 3).

Total Protein

The methods depend upon the combustion/digestion of the organic matter of the sample to release N for chemical reaction and volumetric estimation.

Lassaigne Test

The Lassaigne Test was devised for the qualitative measurement of N, S, and the halogens. Nitrogen detection is based on the digestion/reaction of the sample with heated Na. If N is present, NaCN is formed. This is reacted with $FeSO_4$ to form the hydroxide, which is precipitated. Heating to near boiling allows the ferrocyanide to form, which with acidified $FeCl_3$ forms the familiar blue precipitate, Prussian blue (Equation 1.4).

$$6NaCN + Fe(OH)_2 = Na_4Fe(CN)_6 + 2NaOH$$

$$4FeCl_3 + 3Na_4Fe(CN)_6 = \underset{\text{Prussian blue}}{Fe_4[Fe(CN)_6]_3} + 12NaCl \qquad (1.4)$$

A modified Lassaigne method was developed for N determination in meat products and baby food.[32] The modified method converts the cyanide into thiocyanate with $(NH_4)_2S_2$ in the presence of an excess of ferric ions in acidic medium for colorimetric measurement. The developers state that the simple spectrophotometric method requires a digestion time of only 15 min compared to the lengthy classical digestion of the Kjeldahl method.

Kjeldahl Method

The Kjeldahl N determination, developed in 1883, depends upon the fact that most organic N compounds are converted into $(NH_4)_2SO_4$ when heated with concentrated H_2SO_4; the exceptions are $–NO_2$ and –N=N– groups, which if present in any quantity should be previously reduced to the amine. Digestion is carried out slowly over a microburner in a loosely stoppered digestion flask to avoid losses by splashing. Figure 1.1 shows the specially shaped Kjeldahl digestion flask and the steam distillation apparatus. This classical apparatus, and others shown later, have been reproduced from a school textbook of organic chemistry to show clearly the principles of the method. The digestion products are then quantitatively rinsed into flask C, treated with excess alkali added from funnel B, and the ammonia generated is distilled over into flask D. From there it is steam distilled and condensed *via* the water-cooled condenser E into standard acid in flask F and in "U" trap G. The combined acid from F and G is titrated

to ascertain the yield of ammonia from which the $\%N_2$ (Equation 1.5) and hence the %protein (Equation 1.6) can be calculated, where x = mls standard acid, and w = the weight of sample.

$$\%\text{N} = \frac{x}{w} \times \text{molarity} \tag{1.5}$$

and

$$\%\text{Protein} = \frac{x \times 6.25}{w} \times \text{molarity} \tag{1.6}$$

With the objective of including the "true" protein level on the label, potentially available protein was calculated from N determined by the Kjeldahl method in an experiment to calculate the true protein content and assess the "*in vitro*" protein digestibility of milk-based starting formulae.[33] True protein was calculated as (total N − non-protein N) × 6.25. Non-protein nitrogen (NPN) was determined in the soluble fraction after the protein had been precipitated with trichloroacetic acid (TCA) and centrifuged. Digestibility was measured by direct enzyme digestion (pepsin and pancreatin) and defined as the increase in NPN after enzyme digestion.

Development of the Kjeldahl Method

New methods have been developed to replace the Kjeldahl method because of the necessity of carrying out two assays to find the difference between NPN and

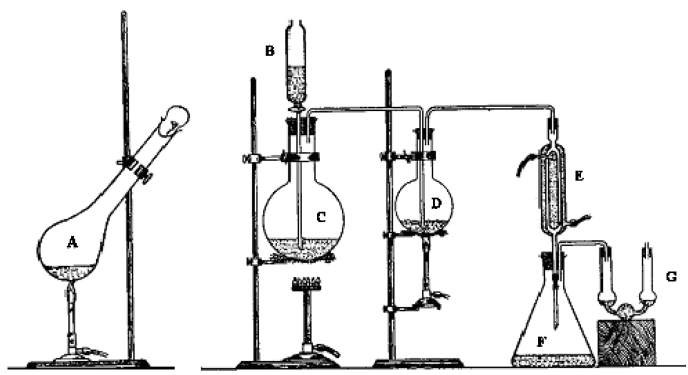

Figure 1.1 *Classical Kjeldahl apparatus.* A. *Digestion flask with a loose fitting stopper over a microburner,* B. *Tap funnel to dispense the 50% NaOH solution into the boiling flask* C *containing the cooled digestion mixture from* A. D. *Steam generator to distil the ammonia.* E. *Water-cooled condenser,* F. *Distillate collection flask containing standard acid, and* G. *"U" trap containing standard acid to retain any airborne ammonia*
(Reproduced from *Organic Chemistry* by F. Sherwood Taylor, William Heinemann Ltd, London, 1949, (first published 1933), (see acknowledgements))

total protein N, and the long time taken by the Kjeldahl digestion, distillation and titration analysis.

A partially automated analyser was described which combined the Kjeldahl digestion with the Berthelot reaction for determining N in biological samples.[34] Any deviation in the results was attributed to the high temperature digestion. The introduction of ion chromatography to determine N as the ammonium ion, to replace the distillation and titration stages of the Kjeldahl method, and to speed up the assay, was developed for food and environmental samples,[35] and the use of microwaves for the efficient digestion of food matrices in an open vessel microwave system was developed by Feinberg *et al.* (1993) to reduce the sample preparation time.[36]

A colorimetric method for measuring N in Kjeldahl digests circumvented the distillation and titration stages and reduced the analysis time.[37] The method was used for nutritional studies on dairy products, dry cereals, cereal-based products, legumes, and cooked food mixtures. The need to analyse large numbers of samples also prompted Yasuhara and Nokihara to develop a colorimetric method for ammonia as an alternative to the Kjeldahl method, which was seen as uneconomical and environmentally unfriendly.[38] The Kjeldahl digest was taken as the starting point for a spectrophotometric method of determining total N, and a sampling rate of 14 per hour was achieved in this case.[39]

Kjeldahl Method Compared to Other Methods

In 1987 the Kjeldahl method was compared to the Hach and Kjeltec methods for a wide variety of samples.[40] The results for total N were in the order of Kjeldahl method > Hach method > Kjeltec method. The Hach method was more sensitive than the others to changes in N content, The Lowry method[41] compared well with the Kjeldahl method for wheat protein determination.[42]

The degree of enzymatic hydrolysis of pea protein using trypsin was controlled using the pH-stat method. The solubility of the hydrolysate was tested at 9 pH values, ranging from 2 to 10, and protein content was determined by three methods, the classical Kjeldahl volumetric method, the Lowry and the modified Lowry methods. There was no agreement between the Kjeldahl and modified Lowry method and no agreement between the Lowry and the modified Lowry method.[43]

The protein content of beans (*Phaseolus vulgaris*) was measured by the micro Kjeldahl, Lowry, Bradford, and biuret methods and the results compared.[44] The micro Kjeldahl method gave different values from the other three methods.

The Kjeldahl method and several spectrophotometric methods have been compared for the determination of total proteins in a range of dried milk powders (listed in Table 1.2 in the order of increasing sensitivity), which might replace the acid digestion and volumetric titration of released NH_3 of the Kjeldahl method.[45]

Spectrophotometric methods are said to be quicker and simpler than the Kjeldahl method, but most of them require a preliminary solvent (lipid) extraction and filtration stage. Samples of milk powder (2 g) were shaken with

Table 1.2 *Range of concentration covered by seven spectrophotometric methods of measuring protein in solvent extracts*
(Reprinted with modification from the *Journal of Food Composition and Analysis*, vol. 16, N.K.K. Kamizake, M.M. Gonçalves, C.T.B.V. Zaia and D.A.M. Zaia, "Determination of Total Proteins in Cow Milk Powder Samples: A Comparative Study between Kjeldahl Method and Spectrophotometric Methods", pp. 507–516, © 2003, with permission from Elsevier)

Method	*Concentration range ($\mu g\ ml^{-1}$)*
1. Biuret-340 nm	2000–10000
2. Biuret-550 nm	2000–10000
3. UV-280 nm	200–1000
4. *p*-Chloranil	30–120
5. Lowry	20–60
6. UV-220 nm	9–40
7. Bradford	1–5

18 ml (2:1 v/v) $CHCl_3$–MeOH for 5 min and the resultant solution filtered. The solvent was discarded and the filtrate re-extracted (shaken) for 5 min with 6 ml $CHCl_3$ and 6 ml H_2O, and filtered. If the solvent layer showed a negative biuret test then the solid was dried in preparation for the spectrophotometric measurement of total protein. The Bradford method[46] gave comparable results to the Kjeldahl method for total proteins in skimmed milk and whole milk powders, without the extraction of lipids. The Bradford method for proteins is based on the reaction with the dye BG-250 since peptides and amino acids do not react.

Collaborative Study of the Kjeldahl Method

The Kjeldahl total N method, AOAC Method 991.20 for total N in milk, was published in 1990 and monitored for 5 more years *via* a multi-laboratory quality assurance program (Lynch *et al.*, 1997).

Dumas Method

Dumas' method is based on the decomposition of compounds to CO_2, H_2O, and gaseous N by heated CuO and a bright Cu spiral, the nitrogen being collected over a solution of KOH. Again the diagram from Sherwood Taylor's *Organic Chemistry* illustrates the method (Figure 1.2)

The organic sample is mixed with an excess of fine CuO and inserted into the 1 m long combustion tube, together with a packing of coarse CuO and a spiral of bright metallic Cu gauze – to decompose oxides of N. The nitrometer contained

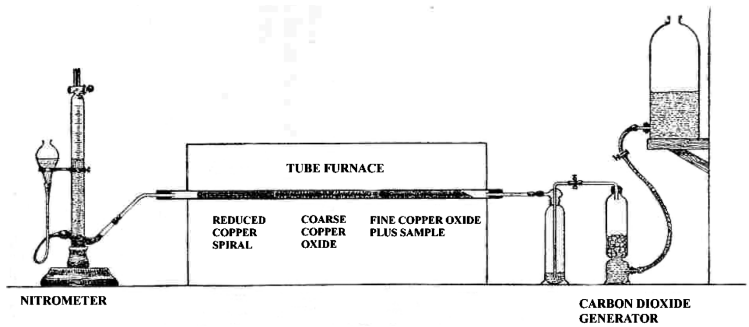

Figure 1.2 *Dumas' method for determining nitrogen. The CO_2 generator is coupled to the inlet of the combustion tube and the outlet from the tube goes to the nitrometer* (Reproduced from F. Sherwood Taylor, *Organic Chemistry*, William Heinemann Ltd, London, 1949. (First published in 1933), (see acknowledgements))

a 40% solution of KOH. CO_2 was used to purge the tube of air while the furnace was heated. Water and CO_2 produced by the combustion of the organic sample were adsorbed or condensed and the N gas was collected in the manometer and the volume recorded and corrected for NTP.

Modern Automated Analysers. The Dumas oxidative combustion system (Foss/Heraeus by Foss Electric (UK) Ltd.) is shown in Figure 1.3, and the schematic form in Figure 1.4.

The ground sample is placed into a small steel crucible, weighed on-line and the crucible placed on the sample chain for insertion midway in the combustion tube. Corundum balls are used as a spacer and the sample is heated by (a) a mobile furnace at 1020 °C traversing from the top of the tube to the sample position for a pre-set time before returning and (b) a static heater at 850 °C at the bottom. The CO_2 carrier gas is supplied at 1.35 bar. The oxygen supply is adjusted (from experience) to suit the composition of the sample and flows directly over the sample while it is being heated. The emerging gaseous mixture is passed through several furnace tubes containing adsorbents and reactants to remove sulphur oxides ($PbCrO_4$), provide post-combustion (CuO/Cu_2O + Pt/Al_2O_3) to complete the oxidation of gaseous products, hydrogen halides and halogens, reduce with Cu any oxides of nitrogen to N, and to remove of any residual oxygen (Cu – reduction tube) and moisture (P_2O_5).

The CO_2/N_2 mixture is measured in Chamber 1 of a thermal conductivity bridge against a reference flow of CO_2 in Chamber 2. The nitrogen content is given by difference.

The LECO FP-428 Dumas combustion analyser was used to measure total N in milk.[47] Suggestions were made for improvements. However, for milk, the Dumas N and Kjeldahl N methods showed good correlation.

The modified automatic Dumas and the traditional Kjeldahl methods were compared for the determination of nitrogen in infant food.[48] The results were

Figure 1.3 *Dumas method instrumentation manufactured by Foss Electric (UK) Ltd.*

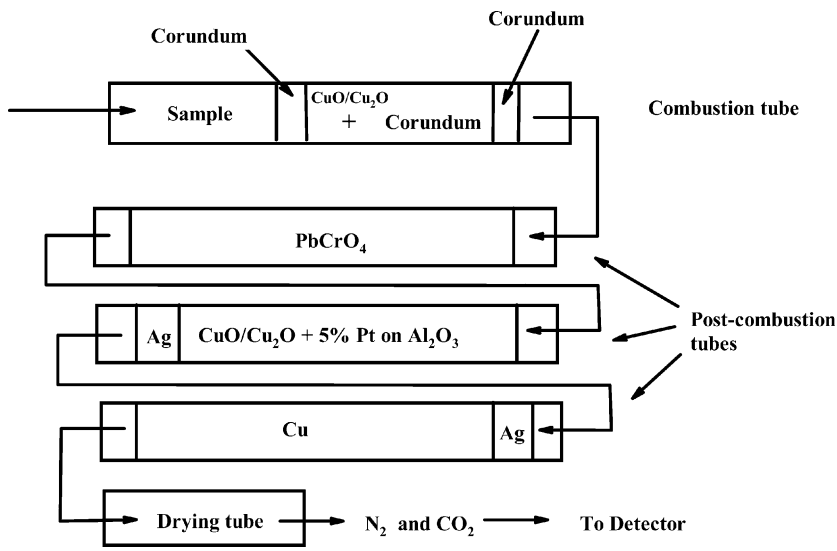

Figure 1.4 *Schematic drawing of the Macro N analyser*
(Configured from the analysis sheets of the Rowett Research Institute, Bucksburn, Aberdeen, Scotland, with permission)

similar but the Dumas method was, *inter alia*, much faster. The two methods were also compared for the application to foodstuffs in general.[49]

Collaborative Studies of Total Nitrogen Methodology

An interlaboratory study (11 laboratories) was conducted to compare the Kjeldahl and Dumas methods for routine analysis of proteins in dairy products (milk, skim milk powder, whole milk powder, whey protein concentrate, infant formula, casein, caseinate, two reference compounds (glycine and EDTA), and a secondary reference (skim milk powder).[50] The two methods gave similar values. The Dumas relative repeatability and reproducibility standard deviations were consistently about 0.35 and 0.75%, while Kjeldahl values declined generally with N content and were significantly larger. The conclusion was that Dumas' method needed Codex Alimentarius status as a recognised test method.

Interlaboratory (15 laboratories) performance was measured for a modified and optimised version of the Kjeldahl method (AOAC 920.123) for the total N determination in different textures of cheese.[51] Crude protein ($N \times 6.38$), g per 100 g levels from 18 to 36% were tested and material homogeneity, size and transfer of sample, recoveries and the modified AOAC method 991.20 were considered. As a result of the statistical data collected on 991.20, the trial directors recommended that the modified method be adopted First Action to replace 920.123 for hard, semi-hard and processed cheese.

Biuret Reaction for Colorimetric Measurement

Biuret (Figure 1.5) is formed from substances containing two or more –NH-CO- groups.

Biuret in the presence of dilute $CuSO_4$ gives a characteristic pink that can be quantified. Proteins and related compounds made strongly alkaline with NaOH, in the presence of dilute $CuSO_4$, give this reaction. In a recent example of the application of the biuret reaction (Arogundade *et al.* 2004)[52] examined the effect of salt type and concentration on the solubility of proteins in defatted *Colocynthis citrullus L.* seed flour, using the biuret method described by Gornall *et al.* (1949).[53]

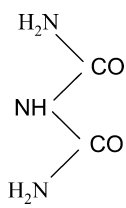

Figure 1.5 *Structure of biuret*

Neutron Activation Analysis (NAA)

NAA has been chosen as an example of a non-destructive remote sensing analyser (Section 3, Andrews and Dallin, 2003). The interest here is in the possible use of this analyser for the virtual extraction or visualisation of N directly in a ground sample in the presence of interference. Early application of NAA to N analysis of corn products experienced problems with activities from other elements in gluten interfering with the induced N activity.[54] Successful separation of the interfering activity led to a method with accuracies equal to the Kjeldahl method. In a recent report of the application of NAA to the measurement of the protein content of ground, oven-dried, homogenised Nigerian foods (soya beans and rice varieties), the nuclear reaction $^{14}N(n,2n)^{13}N$ was used to determine N (and hence protein content) by 511 keV gamma rays.[55] Many other elements can contribute to the intensity of the annihilation peak, and it has not hitherto been considered in the quantitative analysis of the N content of foods. The chief interferences are $^{79}Br(n,2n)^{78}Br$, $^{31}P(n,2n)^{30}P$, $^{31}P(n,\alpha)^{28}Al$, and $^{39}K(n,2n)^{38}K$, and proton recoil reactions from C and O. Once the contributions to the peak at 511 keV had been determined, corrections were applied for each individual reaction, and the total protein data were comparable with those obtained by the Kjeldahl method.

Non-protein Nitrogen

Often it is useful to estimate non-protein N and the Kjeldahl method has been used to measure the nitrogen content of fractions made from foods by various extraction techniques. NPN in pooled sweet and acid wheys was estimated by membrane dialysis at different MW cut-offs, and TCA/phosphotungstic acid soluble N fractions.[56] NPN values varied with membrane porosity, and dialysable N was generally lower than acid soluble N.

Total Carbohydrate

Introduction

Total carbohydrate consists of sugars (mono and oligosaccharides) and polysaccharides (starch and the non-starch polysaccharides; pectin, soluble and insoluble dietary fibre, *e.g.* cellulose and hemicellulose). Total starch (TS) is sub-divided into digestible starch (DS), resistant starch (RS) and dietary fibre (DF). An excellent text on the chemistry of food components covers these topics concisely.[57] The debate about the actual composition of the carbohydrate fraction, the main contention being between the Englyst[58] (enzymatic–chemical) and Prosky (Prosky *et al.*, 1988, Chapter 2, ref. 78) (enzymatic–gravimetric) methods, is about what should and should not be included. This continues,[59,60] and therefore, there is less evidence in the literature of routine carbohydrate analysis and more research and development of analytical protocols, which is discussed under the methods sections elsewhere, *e.g.* biochemical release of dietary fibre, Chapter 2.

Carbohydrate Content by Difference in Proximate Analysis

In a 1990 report, total carbohydrate was calculated as the residue by difference from the total of fat, protein, moisture/solids, ash, and fibre values. A review of collaborative studies of these parameters was made to determine the likely precision of the process. The procedure was judged as having poor precision among laboratories and high variability.[61] Even so, the "by difference" method was used in 2002 for the proximate analysis of Nigerian oil seed,[62] and Menezes *et al*., 2004 averred that most composition databases contain total carbohydrate data calculated by the difference method.

Saccharide Content by GC-MS

Introduction. The food is hydrolysed to prepare a sample for GC-MS analysis. The preparation of the alditol acetate derivative of the neutral mono- and oligosaccharide is popular, but for acidic residues the TMS-methyl derivative is preferred for GC-MS analysis.[63]

Monosaccharides and Oligosaccharides. The use of GC-MS to detect and quantify the volatile derivatives of the hydrolysed saccharides has been the method of choice for many years. A very instructive account of the sample preparation and analysis of alditol acetates by EIMS and CIMS was given by Kamerling and Vliegenthart in 1989.[64] Written from a mass spectrometric point of view, the chapter contains most of the mass spectra identifying the individual sugars and it also explains the fragmentation processes used diagnostically in the interpretation.

The sample preparation for the TMS derivative analysis by GC-MS of honey carbohydrates was to dilute, transfer to the autosampler vial and freeze dry for 4 h.[65]

Polysaccharides. Food grade polysaccharide gums, tragacanth, karaya, ghatti, carob, guar, arabic and xanthan were hydrolysed with TFA and the neutral monosaccharides derivatised for GC-MS. Sample preparation included defatting, starch degradation and protein precipitation.[66]

Starch Content

Introduction. Collaborative research studies by colleagues from CSIC and the Nutrition and Analytical Chemistry Faculties in Madrid have produced valuable working protocols for the analysis of starch fractions.[67,68] The inaccuracy of the "by difference" estimation of carbohydrates in food composition tables prompted them to develop methods for TS and RS.

Total Starch. 2 M KOH was used to dissolve the RS and the total starch was hydrolysed with amyloglucosidase. The released glucose was determined and the TS calculated as glucose $\times 0.9$. Total starch was measured on bread,

spaghetti, rice, biscuits, lentils, chickpeas, beans, frozen peas, boiled potatoes and crisps.

Resistant Starch. The method for RS published as an appendix to reference 68 contains the complete details to perform the assay. Briefly, it involved milling, defatting, and homogenisation as required by the sample matrix. The aliquot for analysis was then buffered (pH 1.5), digested with pepsin under constant shaking to remove protein, re-buffered to pH 6.9, digested with α-amylase to hydrolyse DS, centrifuged and washed (repeat centrifuge and wash) discarding the supernatants; RS was dispersed with KOH with constant shaking, adjusted to pH 4.75 and enzymically hydrolysed to glucose with amyloglucosidase, centrifuged and the supernatants collected, made up to volume, washed and re-buffered. The glucose released was quantified from a standard curve. After a collaborative trial among three laboratories the RS method was applied to rice, spaghetti biscuit, white bread, crispbread, pea flour, lentil flour, corn flakes and All Bran.

Digestible Starch. DS was calculated as the difference between TS and RS.

Applications. An excellent example of the application of modern proximate analytical methods (references 65 and 66) to the improvement in the measurement of carbohydrate fractions (starches) was reported by Rosin *et al.*, 2002.[69] They recognised rapidly digestible starch (RDS), slowly digestible starch (SDSt) and RS, and present methods for the analysis of TS and RS. DS was calculated by difference (TS – RS). RDS and SDSt were calculated from the percentage of 30 and 60 min hydrolysed starch after aliquots taken every 30 min (0 to 180 min) from the α-amylase digestion, further hydrolysed by amyloglucosidase, were used to construct the hydrolysis index.

RS analysis involved removal of protein by incubation with pepsin (40 °C, 1 h at pH 1.5), incubation with α-amylase (37 °C, 16 h at pH 6.9), suspending the sample in 2 M KOH and shaking for 30 min, and incubating with 1 ml (300 U ml^{-1}) amyloglucosidase at 60 °C and pH 4.75 for 45 min. TS, DS and RS values were obtained for whole rice, polished rice, corn, polenta, white spaghetti, white bread, potatoes, peas, beans, lentils and chickpeas.

The same 11 test foods were used in a study by the same group working with Brazilian colleagues to improve the quality of carbohydrate data for nutritional work on Brazilian foods.[70] The nomenclature from the nutritional viewpoint was the indigestible fraction being composed of total, soluble and insoluble fractions. (Summarised from ref. 69 with permission from Elsevier)

Non-starch Polysaccharide Content

Many nutritional groups use the phrase non-starch polysaccharides (NSP) to include all the components known as dietary fibre. In a study of the NSP of Mexican Foods, Sánchez-Castillo *et al.*, 1999,[71] used the method of Englyst *et al.* (1994, Chapter 2, ref. 81).

Figure 1.6 shows a flow diagram of the Englyst method for the release of neutral sugars for GC analysis.

Sample
|
Add 2 mL DMSO; 30 min at 100°C
|
Add 8 mL heat-stable amylase (Termamyl) solution; 10 min at 100°C
|
Add 0.5 mL pancreatin/pullulanase solution
|
30 min at 50°C; 10 min at 100°C
|
Cool to 0°C and add 0.15 mL HCl, 5 mol/L
|
Add 40 mL acidified ethanol; 30 min at 0°C
|
Centrifuge, wash with acidified 85% ethanol;
wash with ethanol; dry with acetone
|
Add 5 mL H_2SO_4 12 mol/L; 30 min at 35°C
|
Add 25 mL water; 1 h at 100°C
|
Hydrolysate → Portion for uronic acids
|
Gas–Liquid chromatography for neutral sugars
|
Add to 1 mL; 0.5 mL internal standard 0.4 mL ammonia solution,
12.5 mol/L 5 µL octan-2-ol; 0.1 mL sodium tetrahydroborate solution
|
30 min at 40°C
|
Add 0.2 mL acetic acid
|
Add to 0.5 mL;
0.5 mL l-methylimidazole; 5 mL acetic anhydride
|
Leave for 10 min
|
Add 0.9 mL ethanol
|
Leave for 5 min
|
Add 10 mL water; 2 × 5 mL KOH, 7.5 mol/L
|
Use top phase for GLC measurement of individual constituent neutral sugars

Figure 1.6 *Flow diagram of the procedure to release neutral sugars for dietary fibre measurement by the Englyst method*
(Reprinted from the *Journal of Food Composition and Analysis*, vol. 12, C.P. Sánchez-Castillo, H.N. Englyst, G.J. Hudson, J.J. Lara, M. de Lourdes Solano, J.L. Munguía and W.P.T. James, "The Non-starch Polysaccharide Content of Mexican Foods", pp. 293–314, © 1999, with permission from Elsevier)

Total Carbohydrate from the Acid Hydrolysate

Total carbohydrate of Nigerian spices was measured by absorbance at 420 nm on the sulphuric acid hydrolysate[72] using the method of Baker and Somers.[73]

Ultrasonics for Non-destructive Proximate Analysis

Mecozzi *et al.* (1999, Chapter 2, ref. 8) described the use of ultrasound-assisted extraction (UAE) at ambient temperature for the hydrolysis of polysaccharides to monosaccharides in the assay for total carbohydrates, and improved upon it by reducing the hydrolysis time further in 2002 (Chapter 2, Mecozzi *et al.*, 2002, ref. 9).

On-line Dialysis/HPLC

When the sugar concentration in a liquid food matrix is at the g l^{-1} level, a rapid automated analysis of individual sugars (and organic acids) using dialysis coupled *via* a flow-switching valve and injection loop to the HPLC has been developed for grape juice, red and white wine, apple juice, and cider (Vérette *et al.*, 1995, Chapter 7, ref. 51). Sensitivity in the g l^{-1} range is readily attainable from these liquid food matrices without the need for additional concentration by SPE.

Automation and Multicomponent Analysis

Remote Sensing

Ultrasonic Velocity Measurement. The notion of extracting compositional information non-destructively from the initial sample was taken a step further with the assessment of fat, moisture, and protein, plus other constituents directly from samples of raw meat mixtures.[74] Ultrasonic velocity measurements were found to increase with temperature in lean tissue and decrease with temperature in fatty tissues. From these values it was possible to predict fat and moisture contents (Figures 1.7 and 1.8).

Under the heading of proximate analysis, the use of ultrasonic velocity measurements to extract information remotely from a ground sample of dry fermented sausage exemplifies the trend towards automation and the circumvention of "wet" chemical methods of extraction. For the food processing industry, such remote data acquisition stations are replacing the need to send samples to the laboratory for analysis.

C, H, N Autoanalyser for Proximate Assays

The measurement of daily nutrient intake requires a method of obtaining proximate analysis on large numbers of samples. The estimation of dietary intake from C, H, N, autoanalyser data has been described.[75] The novel use of the autoanalyser for total fat content was correlated with Soxhlet ether extraction data ($n = 50$; $R = 0.979$; $Y = 0.941x - 0.43$; $p < 0.001$).

NIR for Remote Analysis

Total Lipids and Total Protein. NIR as a rapid and non-destructive method to determine total fat and protein in mixed, homogenised and freeze-dried human

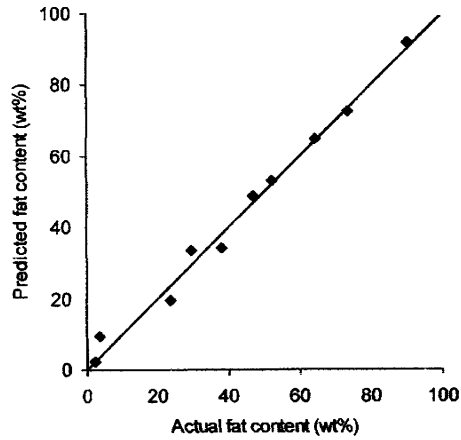

Figure 1.7 *Fat content predicted by ultrasonic velocity measurements against solvent extraction method values*
(Reprinted from *Meat Science*, vol. 57, J. Benedito, J.A. Carcel, C. Rossello and A. Mulet. "Composition Assessment of Raw Meat Mixtures using Ultrasonics", pp. 365–370, © 2001, with permission from Elsevier)

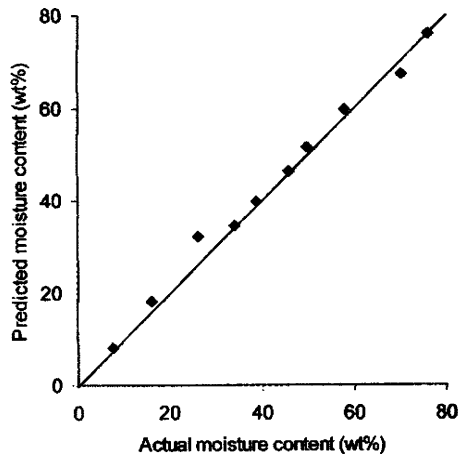

Figure 1.8 *Moisture content predicted by ultrasonic velocity measurements against oven drying method values*
(Reprinted from *Meat Science*, vol. 57, J. Benedito, J.A. Carcel, C. Rossello and A. Mulet. "Composition Assessment of Raw Meat Mixtures using Ultrasonics", pp. 365–370, © 2001, with permission from Elsevier)

diets was described by Almendigen *et al.*, (2000).[76] The new method was compared with the Kjeldahl and Folch methods for total protein and total fat content of student diets. A correlation coefficient of 0.99 against the Folch method for fat was seen as highly accurate and 0.81 for protein against the Kjeldahl method as acceptable; such that both assays for their nutritional work could be replaced

by the quicker and less expensive NIR probe. The method worked well for fat, but accuracy for protein was less convincing.

NIR for Moisture, Oil and Protein Analysis

The GRAINSPEC NIR whole grain analyser (Foss Electric Multispec Division, York, UK), calibrated by the manufacturer, was used to obtain proximate values for moisture, oil and protein content of the initial soybean grain samples in a study (interlaboratory test) of soymilk and tofu.[77]

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