FAO FOOD AND NUTRITION PAPER 14/7

manuals of food quality control

7. food analysis: general techniques, additives, contaminants, and composition

FOOD AND AGRICULTURE ORGANIZATION
OF THE UNITED NATIONS ROME

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7. food analysis: general techniques, additives, contaminants, and composition

prepared by fao with the support of the swedish international development authority (sida)

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
Rome, 1986

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FOREWORD

The control of food safety and quality is an integral part of national programmes for development. National food control systems are designed to protect the health and welfare of the consumer, to promote the development of trade in food and food products, and to protect the interests of the fair and honest food producer, processor or marketer against dishonest and unfair competition. Emphasis is placed on the prevention of chemical and biological hazards which result from contamination, adulteration or simple mishandling of foods. Also important are the maintenance of general food quality and the control of the use of food additives and food processing procedures.

In order to establish a workable food control system, a national government must:

1. Enact food control legislation.
2. Promulgate regulations to enforce that legislation.
3. Create an agency to conduct the enforcement.
4. Establish food inspection and analysis staff within the agency or agencies concerned.
5. Provide physical facilities including a food control laboratory.

To assist the national governments of developing countries in this process, FAO, with the support of the Swedish International Development Authority (SIDA) has published the series Manuals of Food Quality Control. These are incorporated as part of the FAO Food and Nutrition Paper Series No. 14, and include:

No. 14/1	The Food Control Laboratory
No. 14/2	Additives, Contaminants, and Techniques (out of print)
No. 14/3	Commodities (out of print)
No. 14/4	Microbiological Analysis
No. 14/5	Food Inspection
No. 14/6	Food for Export
No. 14/7	Food Analysis: General Techniques, Additives, Contaminants, and Composition
No. 14/8	Food Analysis: Quality, Adulteration, and Tests of Identity

In addition, FAO, WHO and UNEP jointly have published many guidelines and other documents designed to further assist developing countries in forming adequate food control systems. These publications include:

Methods of Sampling and Analysis of Contaminants in Food - A Report of the Second Joint FAO/WHO Expert Consultation, Rome - 1978

Guidelines for Establishing or Strengthening National Food Contamination Monitoring Programmes - FAO Food Control Series No. 5 - 1979

Guidelines for the Study of Dietary Intakes of Chemical Contaminants - WHO Offset Publication No. 87 - 1985

Guide to Codex Recommendations concerning Pesticide Residues, Part 2 - Maximum Limits for Pesticide Residues, Second Preliminary Issue - Rome - 1985

Recommended Practices for the Prevention of Mycotoxins in Food, Feed and their Products - FAO Food and Nutrition Paper No. 10, Rome - 1979

Food Standards, Codes of Practice and Methods of Analysis Recommended by the Codex Alimentarius Commission - Joint FAO/WHO Food Standards Programme (several titles)

Food Additive Evaluations and Specifications of Purity and Identity - Reports and Monographs of the Joint FAO/WHO Expert Committee on Food Additives (several titles)

The above publications, and others, are available to persons and organizations. FAO is also interested in receiving comments regarding this volume and suggestions for future improvement. Please send to:

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FAO wishes to acknowledge the generous support of the Swedish International Development Authority (SIDA), in the preparation of this volume, and the efforts of Mr. J. Weatherwax and Mr. P.G. Martin who were responsible for the preparation of the text.

SPECIAL NOTE

The methods and analytical procedures described in this Manual are designed to be carried out by properly trained personnel in a suitably equipped laboratory. In common with many laboratory procedures, the methods quoted frequently involve hazardous materials.

For the correct and safe execution of these methods it is essential that laboratory personnel follow standard safety procedures for the handling of hazardous materials.

While the greatest care has been exercised in the preparation of this information, FAO expressly disclaims any liability to users of these procedures for consequential damages of any kind arising out of or connected with their use.

The methods are also not to be regarded as official because of their inclusion in this Manual. They are simply methods which have been found by experience to be usable in the average laboratory.

CONTENTS

8. COMPOSITIONAL ANALYSIS METHODS

1. SCOPE OF THIS MANUAL OF FOOD ANALYSIS

This Manual includes discussions of analytical techniques as well as analytical methods generally applicable to all foods. Specific methods used only with certain foods are covered in the Manual, "Food Analysis: General Quality, Adulteration, Identity."

The techniques discussions cover all physical manipulations during analysis as well as how to effectively use analytical equipment, instruments and glassware. Proper technique is all important for analytical results to be consistent, reproducible and valid. The manipulation techniques shown will keep error at a minimum if followed exactly. It is therefore recommended that they be adopted, even if another technique has been previously used.

The general methods are those for additives, contaminant residues, and compositional (proximate) analysis in foods. The following precautions apply to all of the procedures:

1. Use only distilled water or the equivalent. (Deionized water is often suitable).

2. Use the best grade of reagent chemicals available and purify if necessary.

3. Follow the method instructions exactly, as many of the procedures are empirical.

4. Use all laboratory safety procedures and equipment.

The reference citations at the end of appropriate sections gives both the references listed in the text, as well as some general references which may provide background information.

The first edition of this Manual was written in 1977 by Mr. Peter G. Martin presently of Lyne, Martin and Radford, Public Analysts, Reading, Berkshire, England. The present revised edition has been prepared with Mr. Martin's support and assistance by Mr. John R. Weatherwax, retired Laboratory Director for the United States Food and Drug Administration, Los Angeles, California, USA.

2. SAMPLE PREPARATION TECHNIQUES

2.1 SUBSAMPLING

The way in which a sample is taken for analysis is the first of a series of potential sources of error in food analysis. Some liquid foods are reasonably homogeneous, but solid and semi-solid foods are always heterogeneous. It must be assumed that the attribute for which the food is being examined, is unevenly distributed throughout the sample.

The laboratory usually has no control over the field sampling of the food product and must assume that the portion received for analysis is representative of the lot of food sampled. (Note that field sampling is covered in detail in Chapter 3 of FAO Food and Nutrition Paper 14/5, Manual of Food Quality Control #5 - Food Inspection). The laboratory sample as received may be bagged, packaged, tinned or bottled and most often includes multiple units. The laboratory analyst must first decide what type of subsampling should be done on the sample received. The analyst may make the subsamples representative or selective depending on what is to be determined in the analysis. If the analyst merely wishes to prove that something exists without worrying about its relationship to the whole, then a selective sample is taken. An example would be spotty decomposition or mould contamination. The sample could be inspected visually or by odour and only the suspect parts removed for examination. Most analyses, however, involve comparison to a standard for the whole, so that representative portions must be taken.

The taking of a representative sample is obviously the more difficult of the two. A liquid food (e.g. milk) generally need only be well mixed or shaken before subsampling. Semi-solid foods are those containing a solid material plus a large portion of free liquid. Examples include many canned foods. In the event that the solid or the liquid are to be analyzed individually, they are separated using a sieve or filter and individually mixed for subsampling. When both solid and liquid phases are to be analyzed as a unit, it is often advisable to blend or otherwise homogenize the two before subsampling.

Solid samples can be of three general types, namely finely divided (e.g. whole cereal grains or flour), an aggregate (e.g. solid mixtures such as sausage), or a whole unit (e.g. an entire fruit). Finely divided dry products can be mixed for subsampling using commercial portioning equipment such as a Jones Divider, or by spreading the sample over a large surface, quartering with a straight-edge and mixing opposite quarters. The two mixed halves can be recombined and the process repeated one or more times to make the subsample portion even more representative. An aggregate solid sample is probably the most difficult as it consists of different food materials usually with different physical properties. The challenge is to take a subsample having a composition representing an average of the food sampled. This most often requires that the aggregate food be chopped or ground before mixing and subsampling. A discussion of this can be found in Section 2.3. The whole unit sample can be most easily subsampled by taking a representative portion of the food. This could be a quarter of a fruit, a piece of loin from a whole fish or other similar sectioning.

In summary, a selective subsample consists only of suspect portions and ignores the remainder of the sample. A representative subsample, however, must as best possible represent an average of the whole sample.

2.2 COMPOSITING

A composite is defined as an admixture of two or more portions of a substance. A composite is formed by first subsampling two or more portions of the same food. An example would be subsampling several individual cans from the same food lot. These subsamples are then combined and mixed so that a portion taken of the composite would be representative of the whole. A composite is simply a

physical attempt to average the normal variation between individual sample units or portions. It is most useful when the analytical result must be compared to a standard or requirement involving the entire food product.

As the composite is to be representative, the subsamples of the individual sample units must not only be taken correctly (see Section 2.1), but must all be approximately the same size, weight or volume. Given correct subsampling, the only remaining problem is to make the composite reasonably uniform and representative. This may involve chopping and grinding as well as physical mixing.

2.3 CHOPPING, GRINDING, MIXING

The well equipped food analysis laboratory should have a variety of sample preparation equipment including mechanical choppers, mincers, grinders, blenders and a hammer or similar mill.

The type of mechanical processing equipment selected will depend on the food product to be treated. The analyst must also keep in mind that mechanical grinders, mills, etc. usually generate heat during the processing. This can possibly change the sample composition, such as for fatty foods where the heat may be sufficient to partially melt the fat. In such cases, hand chopping and mixing may be the best procedure. In other instances, the sample may have to be frozen before grinding. The analyst must judge the best method for himself, depending on the kind of food and the substance for which it is to be analyzed.

The moisture content of a food also plays an important role in determining the food processing procedure or equipment to use. Dry foods can generally be milled, while moist foods can be chopped, minced or ground. Very moist and liquid foods can be blended. The home food processors now available are very useful for many products.

If no mechanical processing equipment is available, then of course hand processing must be done. The tools used include knives, graters and choppers. When a sample is processed by hand, it must be sufficiently finely divided to permit proper mixing and later subsampling of the mixture.

The analyst must always keep in mind that proper sample preparation is not only to gain a representative portion for analysis, but is also to prevent change in the sample which may result in a biased analytical result.

2.4 FREEZING AND THAWING

Freezing is often the only way to prevent a change in a food before analysis or for reserve storage. Examples include foods for decomposition analysis or foods which were sampled while frozen. It was mentioned in Section 2.3 that some foods must be frozen before grinding or other processing.

The single most important problem in handling frozen food samples is proper thawing before analysis. Thawing must take place in such a manner that the composition of the food remains unchanged. Thawing should be done slowly without heat and in a closed container to prevent moisture loss by drying or gain by condensation. Any separated liquid must be mixed back in the thawed product before subsampling for analysis.

2.5 RESERVE STORAGE

The reserve portion of a food sample must be maintained in storage so that there is very little or no change from the original analysis. Ideally, the reserve portion analyzed at a future time will give a result equivalent to the original.

The storage container is very important. The container should protect the product against moisture loss or gain, and physical damage such as attack by vermin. In some cases the container must be hermetically sealed to prevent air oxidation.

For dry storage at room temperature, glass or metal containers with appropriate closures are usually sufficient. Rigid plastic containers would be a second choice. Plastic and paper bags are not as useful for dry storage because they are easily broached by insects or mechanically. The analyst should also keep in mind that commercially canned foods can deteriorate in storage and should check such stored items periodically.

Glass, rigid plastic, or thick plastic bags can be used for frozen storage. If glass or rigid plastic is used, space must be allowed in the container for expansion of the ice so as not to break the container. If thin plastic bags or paper is used, the product may lose moisture due to drying while frozen.

It is not advisable to store a reserve portion in a refrigerator for any great length of time, unless the container is air-tight. Even if the stored food does not otherwise deteriorate, it may become mouldy unless protected. Preservatives may be added only if they do not affect the desired analysis.

The amount of food product to be kept as a reserve depends on any legal as well as analytical requirements. A reasonable reserve would be that amount necessary to do three analyses. More should be kept if storage space is available.

The above reserve storage precautions also apply to overnight or longer storage while the analysis is being performed.

3. HANDLING TECHNIQUES

3.1 SOURCES OF ERROR

Error is defined in one dictionary as a "....deviation from accuracy or correctness; a mistake...." Most experienced analytical chemists will agree that possible sources of error in analysis seem to be unlimited. On a practical level, however, most error is controllable.

Many texts divide error into two categories, namely random and systematic. Random errors are considered to be unsuspected and nonreproducible errors which are beyond control. Their very randomness, however, permits use of the techniques of statistics and probability to evaluate analytical results. On the other hand, systematic errors are those which arise from definite (and often known) causes and which are controllable. Systematic errors result in bias in the analysis result. This bias can be positive or negative and when considering the effect of a known error it is useful to determine its direction. This is especially true when an attempt is made to find the major source of error which may have caused an incorrect result. For example, if the result is incorrect and is unusually high, look for sources of contributing errors which might be something like a low potency standard which would give a calculated high analytical result.

Systematic error can be grouped into five general sources:

1. **Analyst** - this includes all human error such as incorrect handling technique, calculation blunders and simple errors in judgement.

2. **Equipment and glassware** - includes malfunctions, incorrect calibrations or use, etc.

3. **Reagents and standards** - includes contamination, low potency, interferences, etc.

4. **Environment** - includes the effects of temperature, humidity, light, etc.

5. **Method** - includes specificity, applicability, sensitivity, etc.

Note that all of the above systematic errors are controllable. The key to control, of course, is the analyst. A properly trained analyst who thinks critically about the analysis and who considers and corrects sources of error, will provide the most accurate analytical result.

The following sections (3.2 - 3.6) discuss some of the physical and handling aspects of analysis and controlling possible error.

3.2 WEIGHING AND SOLID TRANSFERS

The first step in most analyses is weighing the sample portion taken for analysis. Even liquid foods are generally weighed rather than portioned volumetrically.

There are two common weighing techniques used in a laboratory. These are direct and by difference. To weigh directly, a container tare weight is determined, the sample is added and the container plus sample reweighed. This gives an accurate sample weight but the major drawback is the necessity to quantitatively transfer the sample from the tared container to another receptacle. This can be done most easily by rinsing using an appropriate solvent. The technique to do this using a glass rod is illustrated in Figure 3.1.

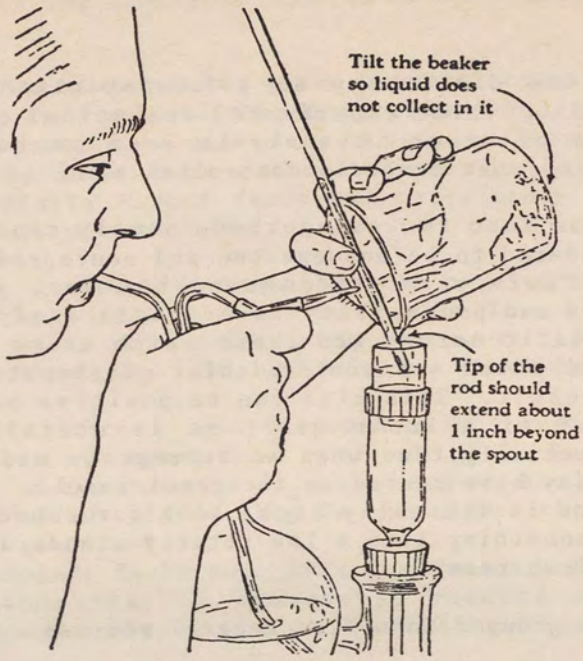

Figure 3.1

The usual method of weighing is by difference. The sample and container are first weighed and then are reweighed after removal of the sample. This is the most convenient procedure and generally does not require quantitative transfer techniques. If, however, the sample is to be transferred to a narrow-neck container such as a volumetric flask, then quantitative rinsing must be done as in Figure 3.2.

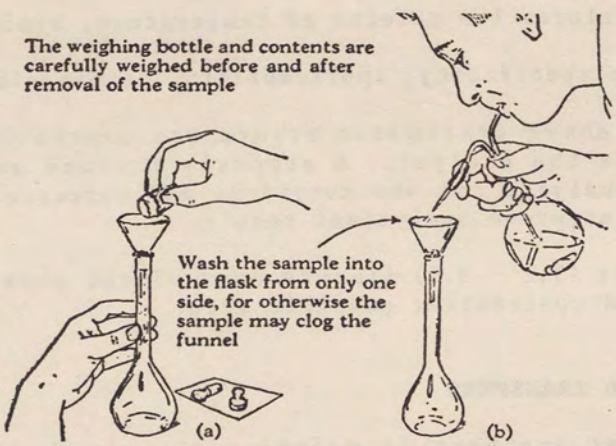

Figure 3.2

3.3 LIQUID AND SEMI-SOLID TRANSFERS

Most liquid transfers are done using volumetric or other pipettes. Pipettes are designed either to deliver a set volume or to contain a volume. Volumetric pipettes are designed to deliver and should never be blown out. The proper technique for volumetric pipettes is illustrated in Figure 3.3. Remember to never pipette by mouth, always use a safety bulb or vacuum tube. Note that drainage time for a grade A pipette is marked on its' side.

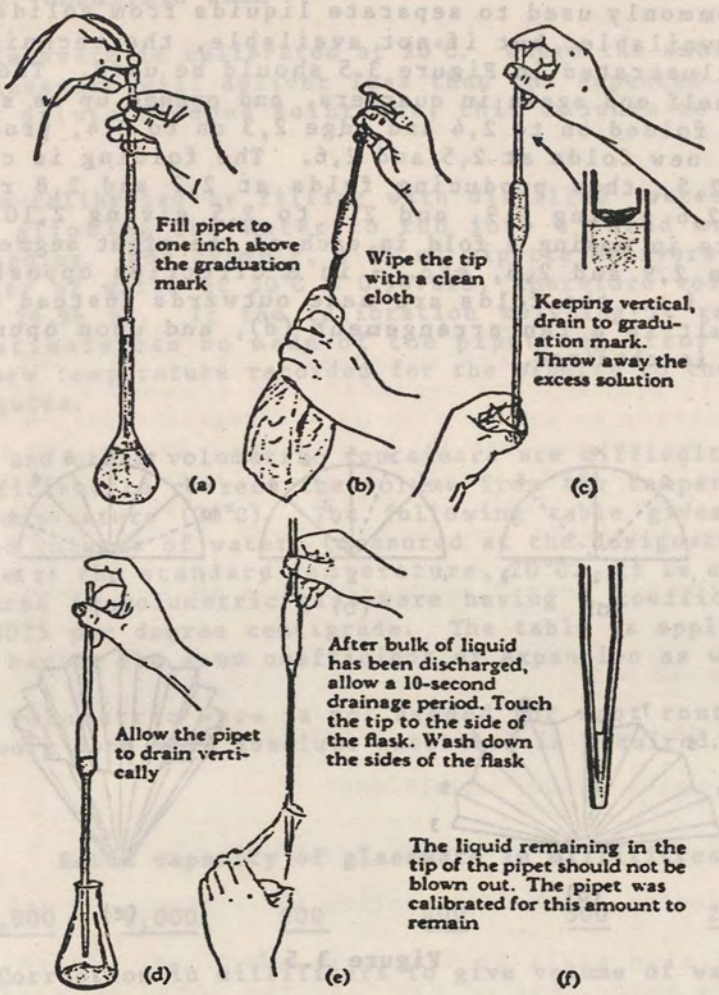

Figure 3.3

Pipettes designed 'to contain' must have the last liquid blown out. These pipettes are not as accurate as volumetric but can be used where only moderate volume accuracy is required. Often they are calibrated in multiple units such as a 10 ml pipette divided into 1 ml increments.

When a liquid transfer is made from an item such as a beaker, then the technique using a glass rod illustrated in Figure 3.4 should be used.

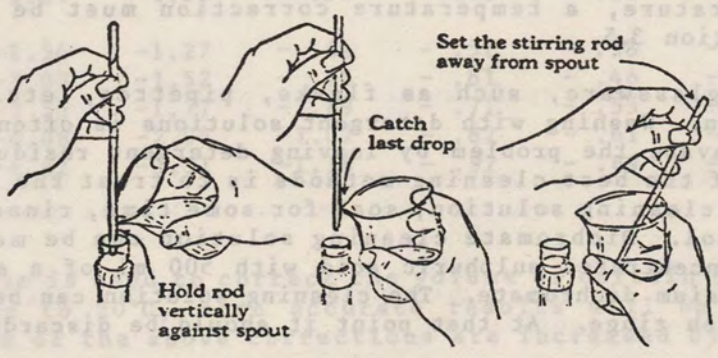

Figure 3.4

Filtration is commonly used to separate liquids from solids. Fluted filter paper is often available, but if not available, the technique to prepare a fluted filter illustrated in Figure 3.5 should be used. The filter paper is first folded in half and again in quarters, and opened up as shown in (a). The edge 2,1 is then folded on to 2,4 and edge 2,3 on to 2,4, producing, when the paper is opened, new folds at 2,5 and 2,6. The folding is continued, 2,1 to 2,6 and 2,3 to 2,5, thus producing folds at 2,7 and 2,8 respectively (b); further 2,3 to 2,6 giving 2,9, and 2,1 to 2,5 giving 2,10 (c). The final operation consists in making a fold in each of the eight segments - between 2,3 and 2,9, between 2,9 and 2,6, etc. - in a direction opposite to the first series of folds, i.e., the folds are made outwards instead of inwards as at first. The result is a fan arrangement (d), and upon opening, the fluted filter paper (e) is obtained.

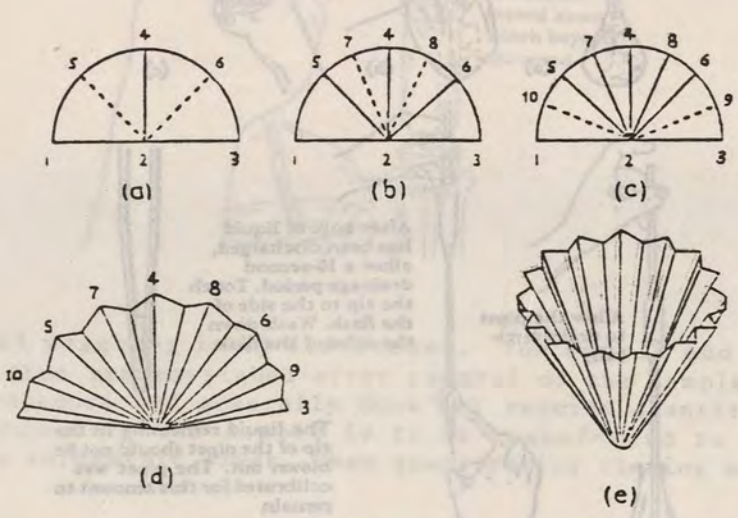

Figure 3.5

3.4 USE OF VOLUMETRIC WARE

The proper use of volumetric pipettes was discussed in Section 3.3. Burettes and their use will be discussed in Section 4.5.

The volumetric flask is indispensible for accurate analtyical work, but only if properly used and cleaned. When filling a volumetric flask, avoid incorporation of air and resultant foaming. Fill until the bottom of the meniscus rests on the calibration line. For most accurate work, remember that the volumetric flask was calibrated at 20°C, so that if it is filled at a different temperature, a temperature correction must be made. This is discussed in Section 3.5.

All volumetric glassware, such as flasks, pipettes, etc., must be kept scrupulously clean. Washing with detergent solutions is often not enough, and in fact can aggravate the problem by leaving detergent residues if not rinsed properly. One of the best cleaning methods is to treat the volumetric ware with dichromate cleaning solution, soak for some time, rinse with distilled water then alcohol. Dichromate cleaning solution can be made by carefully mixing 800 ml concentrated sulphuric acid with 500 ml of a saturated aqueous solution of potassium dichromate. The cleaning solution can be reused until it develops a greenish tinge. At that point it should be discarded.

Another, less rigorous, cleaning solution is 15% trisodium phosphate in water. A warm solution of this (about 70°C) has been shown to be very effective.

3.5 CALIBRATION OF VOLUMETRIC WARE

Most volumetric glassware is calibrated at 20°C. Where the ambient temperature is higher, such glassware will deliver less than the expected weight. In the case of water and dilute aqueous solutions, this amounts to about 0.02% per degree.

Pipettes may be re-calibrated by filling with distilled water at the desired temperature, and allowing the water to run into a tared weighing bottle, capping and re-weighing. For example, a 25 ml pipette delivers 24.8266 g water at 30°C; the density of water at 30°C = 0.987623, therefore volume delivered = 24.8266/0.987623 = 24.96 ml. If the calibration weighing is repeated three or more times, an estimate can be made of the pipetting error and the volume delivered at the new temperature recorded for the pipette to the correct number of significant figures.

Volumetric flasks and other volumetric containers are difficult to recalibrate. It is usually sufficient to correct the volume from the temperature of use to the calibration temperature (20°C). The following table gives the correction to various observed volumes of water, (measured at the designated temperatures) to give the volume at the standard temperature, 20°C. It is assumed that the volumes are measured in volumetric glassware having a coefficient of cubical expansion of 0.000025 per degree centigrade. The table is applicable to dilute aqueous solutions having the same coefficient of expansion as water.

Recalibration of volumetric ware is not needed for most routine work and in fact, is usually only done when absolute exactness is required.

Measurement Temperature	**Rated capacity of glassware in millilitres at 20°C**						
(°C)	2,000	1,000	500	400	300	250	150
	(Correction in milliliters to give volume of water at 20°C)						
15	+1.54	+0.77	+0.38	+0.31	+0.23	+0.19	+0.12
16	+1.28	+ .64	+ .32	+ .26	+ .19	+ .16	+ .10
17	+ .99	+ .50	+ .25	+ .20	+ .15	+ .12	+ .07
18	+ .68	+ .34	+ .17	+ .14	+ .10	+ .08	+ .05
19	+ .35	+ .18	+ .09	+ .07	+ .05	+ .04	+ .03
21	- .37	- .18	- .09	- .07	- .06	- .05	- .03
22	- .77	- .38	- .19	- .15	- .12	- .10	- .06
23	-1.18	- .59	- .30	- .24	- .18	- .15	- .09
24	-1.61	- .81	- .40	- .32	- .24	- .20	- .12
25	-2.07	-1.03	- .52	- .41	- .31	- .26	- .15
26	-2.54	-1.27	- .64	- .51	- .38	- .32	- .19
27	-3.03	-1.52	- .76	- .61	- .46	- .38	- .23
28	-3.55	-1.77	- .89	- .71	- .53	- .44	- .27
29	-4.08	-2.04	-1.02	- .82	- .61	- .51	- .31
30	-4.62	-2.31	-1.16	- .92	- .69	- .58	- .35

If the above table is used to correct the volume of certain standard acid and alkali solutions to 20°C, more accurate results will be obtained if the numerical values of the above corrections are increased by the percentages given below:

Solution	Normality		
	N	N/2	N/10
Nitric acid	50	25	6
Sulphuric acid	45	25	5
Sodium hydroxide	40	25	5
Potassium hydroxide	40	20	4

3.6 CARE AND USE OF STANDARDS

The term 'standards' includes all reagents and materials used as a reference and against which samples are measured. There are two broad classes of standards, namely primary and secondary. A primary standard is one whose composition and purity is established by a recognized national or international authority. (Examples are the U. S. National Bureau of Standards and the European Community Bureau of Reference). Primary standards are often very expensive and/or very difficult to obtain. Therefore, many laboratories make do with secondary standards and compare these to the primary where possible. Secondary standards are usually the daily working standards of the laboratory and most often are the purest form available to the laboratory on a routine basis.

All standards should be:

1. Stored separate from other reagents and materials.

2. Stored under proper conditions.

3. Used in a manner to prevent contamination.

4. Periodically checked to confirm their stability and purity.

The above would also apply to standard solutions. These requirements are basically common sense, but unless they are complied with, a standard may quickly lose its validity. Separate storage is needed to control and identify which materials are standards and which are not, so that everyday reagents are not accidentally used as reference standards. Proper storage is obviously necessary to prevent early deterioration. Proper use includes using clean implements and never returning unused standard to the container. Periodic checking is the final and probably most important requirement. Short lived organic materials have to be frequently checked. Even extremely stable inorganics and metals should be checked on a routine (though infrequent) basis.

3.7 TEXT REFERENCES

None

Further Reading

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ISO TC48 has published over fifty specifications for laboratory glassware and related apparatus.

4. GENERAL ANALYSIS TECHNIQUES

4.1 HEATING AND DRYING

Heating devices commonly used in a laboratory include:

Localized heating	Overall heating
Hot plates	Gravity convection oven
Mantles	Mechanical convection oven
Steam baths	Vacuum oven
Water baths	
Burners	
Heating tape	

Two aspects are important when using a localized heating device, spot overheating and accurate temperature control. All electrical heating devices are, or can be, rheostatically controlled. Therefore, they are useful over a broad range from warming reaction vessels to distillations. Baths, however, are usually limited to evaporation duty as they have no flexibility. Burners should only be used when nothing else is available (and flammable solvents are not in use), or when extremely hot local heating is required (such as charring before ashing).

Ovens are indispensable for uniform sample drying by heat. Some precautions must be observed, however, to ensure best operation:

1. Do not overload an oven. Oven efficiency decreases dramatically when it is charged with too many units at one time.

2. Do not try to dry extremely wet materials. It is usually best to evaporate on a steam bath first, then use the oven.

3. Check (and record) the oven temperature frequently to ensure it is operating correctly.

4. Never leave the oven door open for any great length of time. If this happens, close the door and re-equilibrate the temperature before use.

5. Ensure that the oven conforms to an appropriate performance standard, e.g. B.S. 2648(1).

The other means of laboratory drying is by use of desiccators. Self-indicating silica gel is probably the most convenient desiccant. Once pink, it should be dried in the oven until blue again. Stronger desiccants such as concentrated sulphuric acid and phosphorus pentoxide may be required on occasion. Magnesium perchlorate is not only a powerful desiccant but also a powerful oxidising agent and must therefore be used with proper precautions.

Desiccator lids without taps must be left slightly ajar when hot crucibles, etc. are put in the desiccator, otherwise a partial vacuum is formed as the crucibles cool. The lid may then become unremovable for many hours. Where the lid has a tap, this must be opened extremely carefully to prevent dispersal of the material to be weighed. Light and friable ash must be in a closed receptacle to avoid this.

4.2 ASHING AND DIGESTION

Ashing is the process of removing organic matter by heat (usually in a muffle or other furnace), to leave a inorganic residue. This is done either to measure the total inorganics or to simplify analysis of trace materials such as heavy metals.

Specific ashing procedures are covered within individual analytical methods. However, there are some general comments and precautions:

1. Dry ashing is applicable to the determination of most common metals in organic matter, excepting volatile metals like mercury. Substances amenable to this method must be charred slowly and the carbon oxidised gently and completely. Loss of metals by volatilisation or by combination with the material of the container must be avoided by working at the lowest possible temperature. Particular care must be exercised when large amounts of halogens are present in either covalent or ionic form. It has been reported that losses of certain metals (e.g. zinc, tin or antimony) occur when dry ashing is carried out in the presence of halides; such losses can be minimised by ensuring that an alkaline ash remains, for example by adding chalk.

2. Dry ashing usually requires little attention. Larger amounts of material can be dealt with more conveniently than by acid digestion by repeated addition of fresh material to already ashed material and re-ashing. Lower blank values are generally achieved than with acid digestion.

3. The dry ashing procedure is of particular advantage when the use of sulphuric acid is objectionable. For instance, for the determination of lead in materials containing appreciable quantities of the alkaline earths, whose sulphates occlude lead sulphate.

4. One of the problems with dry ashing is that it is sometimes difficult to obtain complete extraction of the metal being determined from some ignited residues. Excessive heating also makes a number of metallic compounds insoluble (e.g., those of tin). Some flour products may give a dark melt in which carbon particles are trapped and will not burn.

5. The slow ignition of some organic materials can cause the evolution of poisonous or noxious fumes, so all ashing operations must be carried out in well ventilated fume hoods.

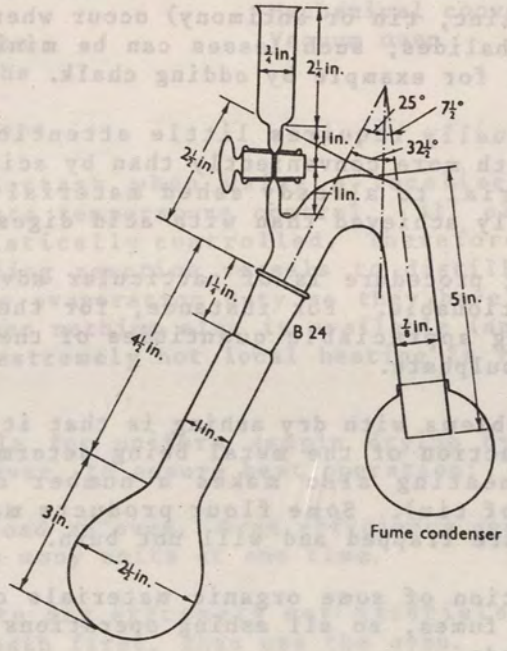
Ashing may be conducted with or without an ash-aid, depending on circumstance. Ash-aids include 50% magnesium nitrate solution or concentrated sulphuric acid. They are used to prevent volatilization of constituents of interest during the ashing process. Individual methods will state how and when ash-aids are to be used.

Acid digestion is the other means commonly used to destroy organic matter in order to determine an inorganic constituent. Sulphuric and nitric acids are usually the digestion acids of choice, sometimes in conjunction with a catalyst. In special cases, perchloric acid is also used, but is very dangerous to work with and safety precautions must be followed closely (2).

One difficulty with acid digestion for metal residue analysis is the likelihood of contamination. All glassware must be previously cleaned with sulphuric and nitric acids and rinsed thoroughly with distilled water before use. All acids and other reagents must be free of the analyte metal. Some of the more commonly used reagents are now available in grades suitable for food analysis. They should not be transferred from the lead-free-glass bottle in which they are supplied. Even when these reagents are used, reagent blank determinations will be necessary. No separate instructions are given in individual methods for reagent blanks, but the blanks must be prepared with the same quantities of reagents as are used in the tests.

When organic matter is heated with mixtures of concentrated acids in Kjeldahl flasks experience has shown that decomposition will take place most efficiently when some means for partial reflux of the hot acids is provided, as by an extension to the neck of the flask. When many oxidations are in progress at the same time, it is advisable to trap the fumes, dilute them with water and dispose of them down the drains rather than into the atmosphere. The following

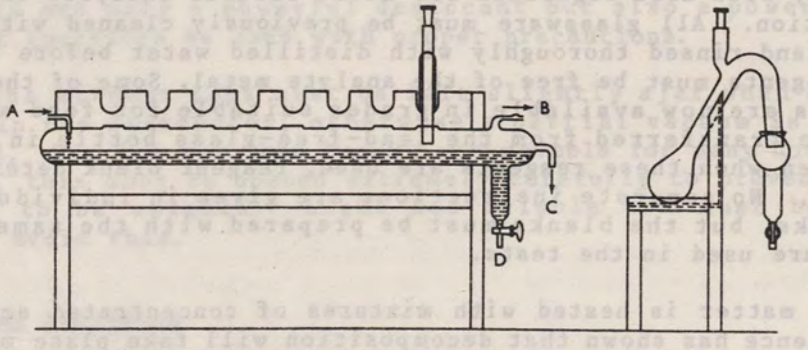
description of suitable apparatus is given for guidance only: Kjeldahl flasks - These should be made of borosilicate glass (100 to 250 ml nominal capacity) fitted with an extension to the neck by means of a standard ground joint. The extension serves to condense fumes into an acid-fume condenser and carries a tap funnel through which the reagents are introduced (see Figure 4.1). Each flask should be supported in a circular hole in a ceramic or other sheet, and the hole should be of such a diameter that the flask receives no direct heat from the burner above the level of the acid.



Modified Kjeldahl flask (open type).
Dimensions are for a flask of 150 ml capacity

Figure 4.1

In the Kjeldahl digestion rack and acid-fume condenser (Figure 4.2), a current of water is kept flowing through the condenser. Removal of acid fumes can also be assisted by connecting the upper outlet to a water pump. Gas heating is preferable. The flasks, not being rigidly clamped, are easily handled when it is necessary to deal with vigorous reactions or excessive frothing, by means of tongues or suitable finger-guards.



Acid-fume condenser and modified Kjeldahl flask. A, water inlet; B, connection to pump; C, wide outlet to waste; D, tap with wide outlet to prevent clogging with solid matter

Figure 4.2

4.3 EXTRACTION

All extractions, whether liquid-liquid or liquid-solid, are partitionings of a material between two phases. In a liquid-liquid extraction, the partitioning is governed basically by the relative solubility of the extractant in the two liquids. In a liquid-solid extraction, this is complicated further by possible physical occlusion of the extractant within some inert solid material.

Most liquid-solid extractions are exhaustive, where a material is to be completely extracted. An example would be fat from a meat sample. Continuous extractors such as a Soxhlet unit (see Figure 4.3) can be used. When using a continuous extractor, the solid sample must be finely divided (to prevent occlusion) and sufficient extractive solvent must be used to provide a good siphoning action plus enough in the reservoir flask to prevent going to dryness. However, if too fine, such as full-cream milk powder, channeling can occur through the mass and extraction may be incomplete.

There are continuous liquid-liquid extractors, but the usual laboratory extractions are done using separatory funnels. As at least one of the liquids in a separatory funnel is likely to be volatile, there may be internal pressure build-up during the extraction process. This should be normally vented through the funnel tip after inversion unless the method specifies the top.

If emulsion formation is a problem, it is often sufficient to gently invert the funnel several times. Partitioning of the extractant between the two liquids takes place at the interface and the mixing of the two liquids does not have to be violent for partitioning to take place.

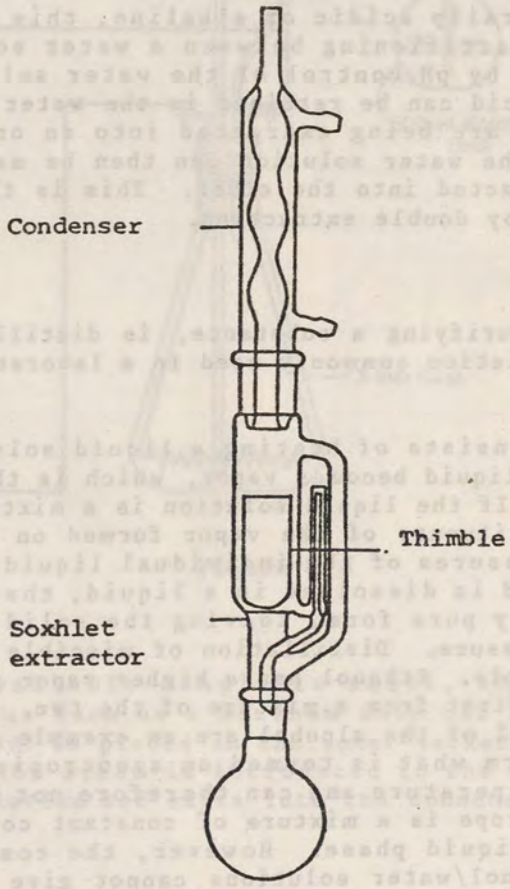
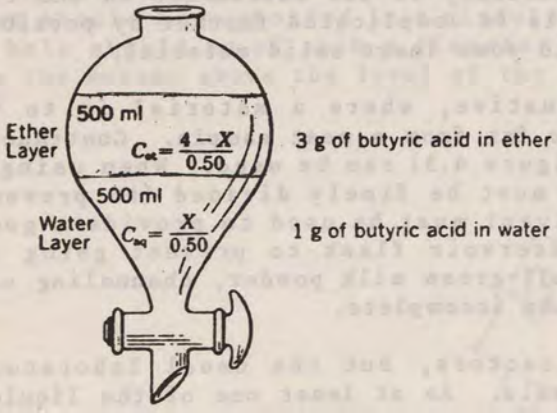


Figure 4.3

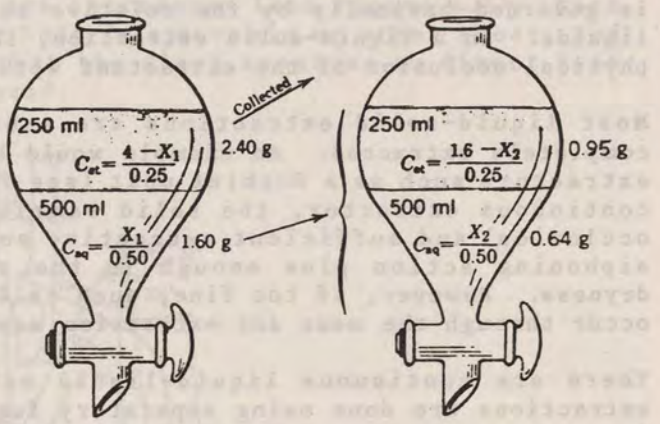
Partioning of a substance is rarely complete in one extraction. For this reason multiple extractions are often necessary for quantitative results. This can be illustrated by the partitioning of butyric acid between water and ether. If 4 gm of the acid were dissolved in 500 ml of water and extracted once with 500 ml ether, then the acid would partition 3 gm into the ether with 1 gm

remaining in the water (see Figure 4.4). If, however, two extractions were done using two 250 ml portions of ether (to total 500 ml), then a total of 3.36 gm of acid is partitioned into the ether (see Figure 4.5).



SINGLE EXTRACTION

Figure 4.4



DOUBLE EXTRACTION

Figure 4.5

Therefore, by using the same total extraction volume in a series of extractions, rather than all at once, more quantitative results can be obtained.

If a substance is naturally acidic or alkaline, this feature can be used to affect the degree of partitioning between a water solution and an organic solvent. This is done by pH control of the water solution. For example, a water soluble organic acid can be retained in the water by making it alkaline, while other materials are being extracted into an organic solvent such as ether. When desired, the water solution can then be made acidic, freeing the organic acid to be extracted into the ether. This is the basis for removal of interfering substances by double extraction.

4.4 DISTILLATION

Another technique for purifying a substance, is distillation. There are three general types of distillation commonly used in a laboratory, simple, steam and fractionation.

Simple distillation consists of heating a liquid solution to a temperature where a portion of the liquid becomes vapor, which is then condensed on a cold surface and collected. If the liquid solution is a mixture, it is important to remember that the constituents of the vapor formed on heating will depend on the relative vapor pressures of the individual liquid mixture constituents. For example, if a solid is dissolved in a liquid, the liquid can usually be distilled in relatively pure form, leaving the solid behind because of its extremely low vapor pressure. Distillation of miscible liquids such as dilute spirits is another example. Ethanol has a higher vapor pressure than water and therefore will distill first from a mixture of the two. However, ethanol-water mixtures containing 95.6% of the alcohol are an example of the phenomena of co-distillation. They form what is termed an azeotropic mixture and will co-distill at the same temperature and can therefore not be separated by simple distillation. An azeotrope is a mixture of constant composition, the same in the vapour as in the liquid phase. However, the composition is pressure-dependent. Dilute ethanol/water solutions cannot give rise to a vapour more concentrated than 95.6% ethanol. However, as distillation proceeds, the

concentration of ethanol in the vapour will exceed that in the liquid until all the ethanol is removed and the boiling-point of the liquid has risen to that of pure water.

In the case of two immiscible liquids, they both contribute the vapour pressure that they would if the other liquid was absent. Thus this vapour pressure will rise to that of the surrounding atmosphere at a lower temperature than would the vapour pressure of either liquid alone. This is the basis of the useful separation technique of steam distillation, under which even solids such as benzoic and sorbic acids can be made sufficiently volatile to distil. A typical steam distillation unit is shown in Figure 4.6. It consists of a steam generator, such as a large flask with a wire heater, a sample flask and a condenser. The amount of steam generated must be carefully controlled to prevent excessive foaming in the sample flask. The long glass tube in the generator flask serves to equalize minor pressure changes, and should extend about three feet above the flask.

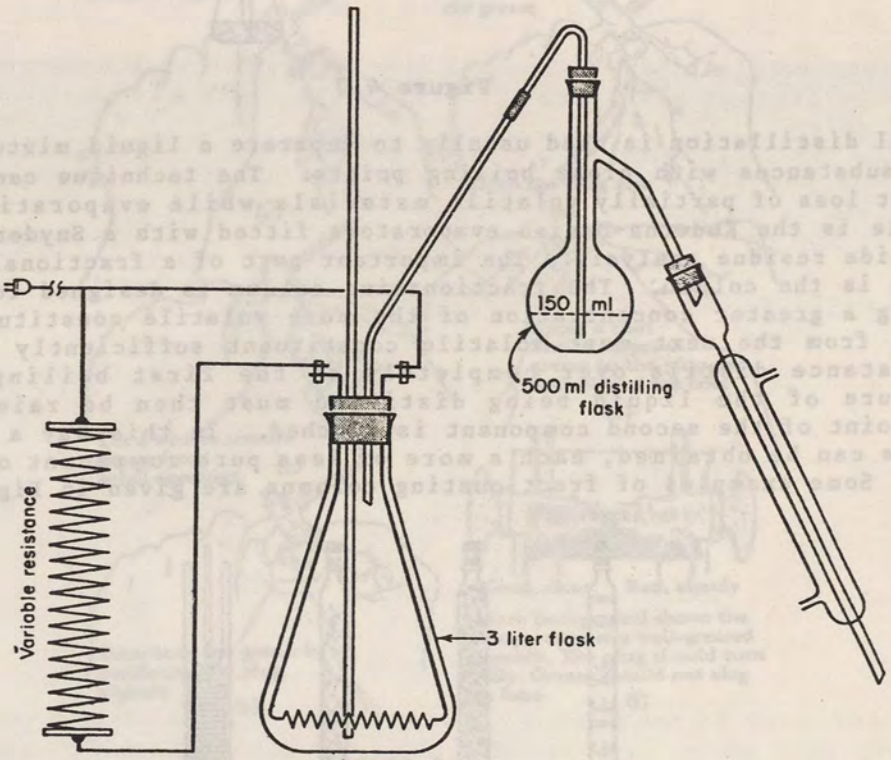


Figure 4.6

If the amount of available sample is small, then a semi-micro steam distillation apparatus such as a Markham unit can be used (see Figure 4.7). The sample (in solution) is placed in the inner jacket via the funnel, which is then stoppered and the steam is introduced to the outer jacket. The steam passes through the solution and exits into the condenser.

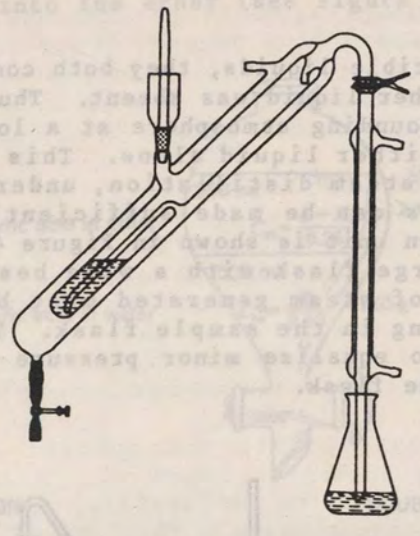


Figure 4.7

Fractional distillation is used usually to separate a liquid mixture having two or more substances with close boiling points. The technique can also be used to prevent loss of partially volatile materials while evaporating a solvent. An example is the Kuderna-Danish evaporators fitted with a Snyder column, used in pesticide residue analysis. The important part of a fractional distillation apparatus is the column. The fractionating column is designed to allow vapour containing a greater concentration of the more volatile constituent to become separated from the next most volatile constituent sufficiently well that the pure substance distils over completely at the first boiling-point. The temperature of the liquid being distilled must then be raised until the boiling-point of the second component is reached. In this way a succession of fractions can be obtained, each a more or less pure component of the initial mixture. Some examples of fractionating columns are given in Figure 4.8.

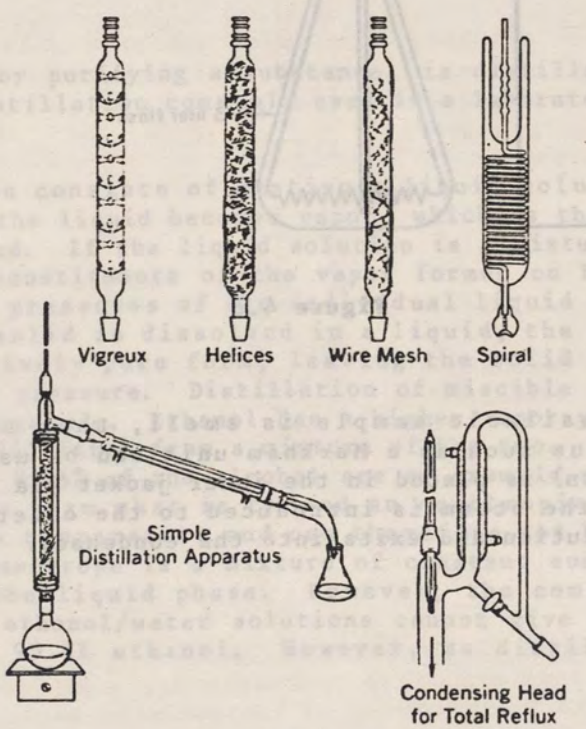


Figure 4.8

4.5 TITRATION

Titrations are among the most frequently performed of analytical techniques in a laboratory. Consistent and accurate results depend on proper care of the burette, correct technique during the titration and accurate reading of the burette.

The burette can be cleaned using dichromate cleaning solution by inverting the burette into a beaker of cleaning solution and applying a vacuum to the burette tip to draw the solution into the burette. Use the stopcock to stop the process when the burette is full. When clean the burette should deliver a water solution without drops adhering to the inner surface.

Some burettes now have teflon stopcocks. These must not be greased, unlike ground glass stopcocks which must be periodically cleaned and greased as illustrated in Figure 4.9.

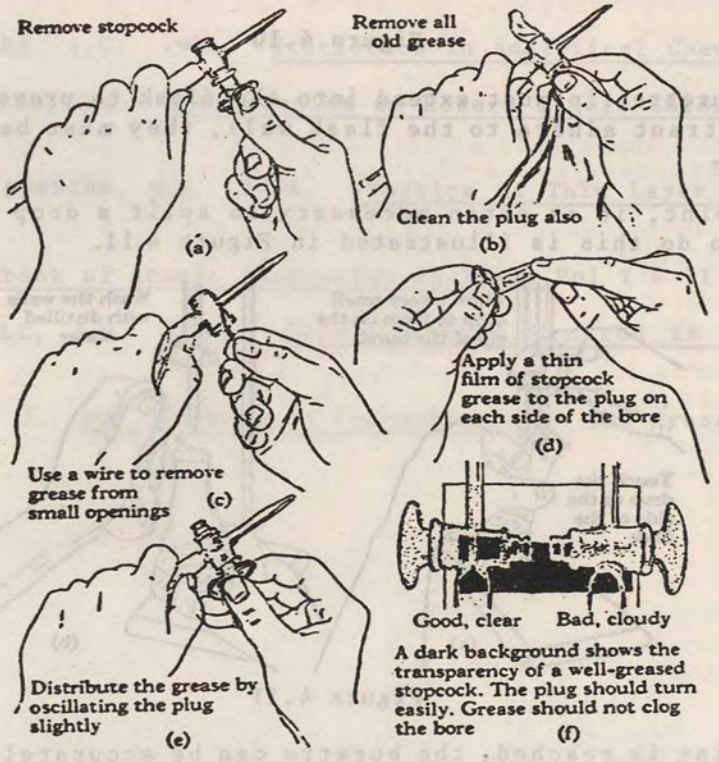


Figure 4.9

Titration technique is simple and straightforward but needs practice to perfect. The stopcock is manipulated with the hand around the barrel of the burette. This permits the analyst to keep the stopcock seated by tugging gently while turning. The other hand swirls the receiving flask. This is pictured in Figure 4.10.

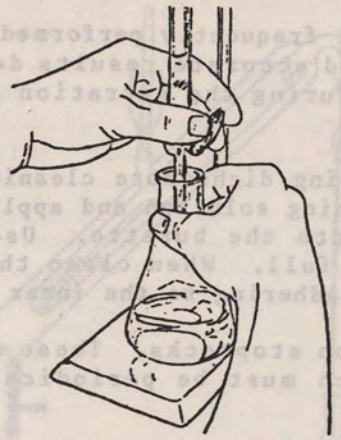


Figure 4.10

Note that the burette tip must extend into the flask to prevent spattering. If any drops of titrant adhere to the flask wall, they must be washed down with distilled water.

Near the end point, it is often necessary to split a drop from the burette. The technique to do this is illustrated in Figure 4.11.

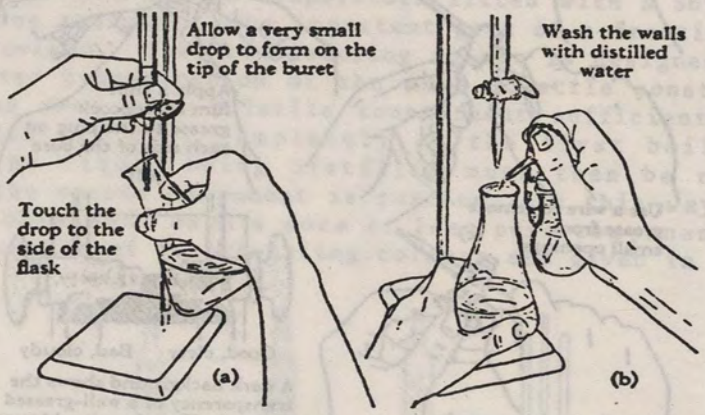


Figure 4.11

When the endpoint is reached, the burette can be accurately read by use of a black strip on a white card as background. This is illustrated in Figure 4.12.

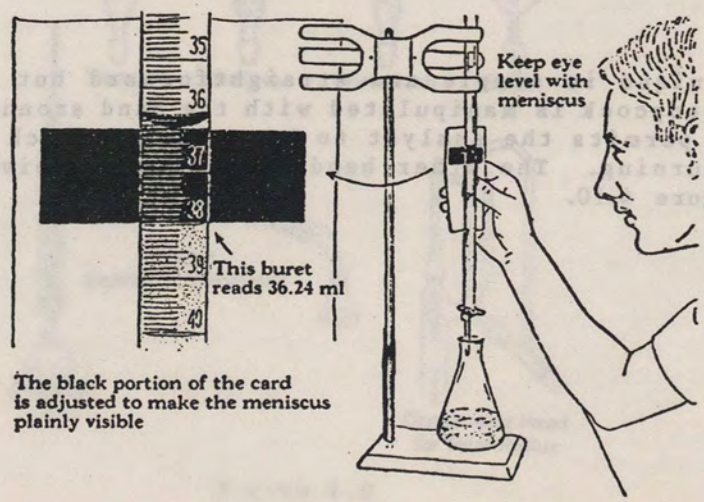


Figure 4.12

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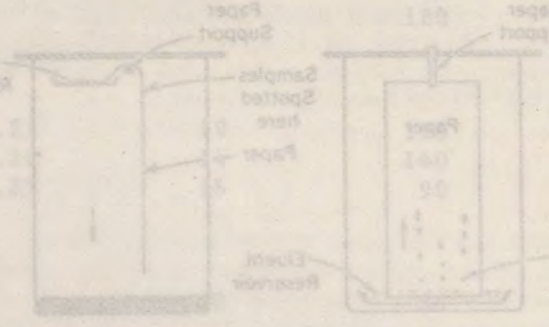


5. DETERMINATIVE TECHNIQUES

5.1 PAPER CHROMATOGRAPHY (PC)

Paper chromatography is a type of partition chromatography, so called because substances are separated according to their solubility between the aqueous phase bound to the paper (the stationary phase) and the water-insoluble organic phase (the mobile phase) which travels up (or down) the paper. Alternatively, the paper can be completely dried and impregnated with a non-polar substance such as liquid paraffin or olive oil, and an aqueous solvent used as mobile phase. This is called "reverse-phase" chromatography.

If the atmosphere of the laboratory is very dry the residual moisture on the paper may be so low that the analyte cannot distribute itself efficiently between the two phases. Separation will not be optimal if equilibrium is not attained. For this reason, non-polar solvents are usually the mobile phase. Also, before chromatography, the paper can be held for several hours in an atmosphere saturated with the vapour of the water-rich phase so that it attains equilibrium. Another method is to moisten the paper with a fine spray or hold it in steam for a few minutes.

Polar organic compounds such as ethylene glycol or dimethyl-formamide may also be used as stationary phases instead of water or aqueous mixtures. Not all separations by paper chromatography rely on partition. for example, some colours can be separated using a 2.5% sodium chloride solution as solvent. In this case the colours are retarded in differing degrees by absorption on the cellulose.

Paper chromatography can be done with the mobile phase either ascending or descending. Figure 5.1 illustrates this showing ascending chromatography in (a) (mobile phase moves up by capillary action) and descending in (b) (mobile phase moves down by gravity).

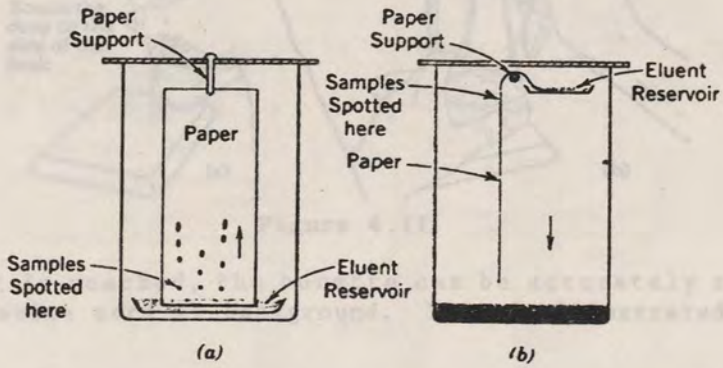


Figure 5.1

In this section we will discuss types of paper and solvents used as well as techniques of sample application and development.

Selection of Paper

Whatman paper no. 1 or equivalent is normally used. Faster development is obtained on papers such as Whatman no. 4 or Schleicher-Schull no. 40a. Preparative work may be carried out on Whatman 3MM, Schleicher-Schull no. 2071, etc. Some properties of the more important chromatographic papers are as follows (taken from Hais and Macek (1)):

Paper	Thickness (mm)	Rate of Movement (hours)(*)	Weight (g/m^2)	Characteristics
Whatman				
No. 1	0.16	15 - 16	85 - 90	Standard paper
No. 2	0.18	17	95 - 100	Standard paper
No. 3	0.36	11	185	Preparative paper
No. 4	0.19	9	90 - 95	Fast
No. 3MM	0.31	11	180	Preparative paper
No. 31ET	0.50	4	190	Very fast
No. 540	0.15	17	85 - 90	Washed, hardened
Schleicher-Schull				
Nr. 2040a	0.18-0.19	7	85 - 90	Fast
Nr. 2040b(M)	0.22-0.24	12	120 - 125	Medium
Nr. 2043a	0.18-0.19	20	90 - 95	Standard Paper
Nr. 2043(MG1)	0.22-0.24	15	120 - 125	Standard Paper
Nr. 2071	0.65-0.70	23	600 - 700	Preparative paper
Filtrak-Nieder-Schlag				
FN 1		9	90	Fast
FN 2		15	125	Standard paper
FN 3		16	90	Preparative paper
FN 8		9	180	Preparative paper
Ederol				
Nr. 202		16	120	Standard paper
Nr. 208		26	120	Slow
Nr. 225			180	Preparative paper
Macherey-Nagel				
Nr. 2212	0.21	19	120	Washed
Nr. 214	0.28	14	140	Standard paper
Nr. 260	0.25	16	90	Standard paper
Leningradskaya				
bumaga B		18	85	Standard paper
Munktell				
Chr 100		9	95 - 100	Standard paper
ELE 130		9	130	Standard paper
Eaton Dikeman				
No. 048	0.18		88	Very fast
No. 248	0.18		88	Standard paper
No. 613	0.14		70	Standard paper
No. 320	2.54		700	Preparative paper

(*) Descending chromatography using (4+1+5) butanol-acetic acid-water.

Selection of Solvent

The following table gives a list of solvents in order of polarity, starting with the most polar (water), and proceeding to the least (paraffin oil):

Solvent	Solubility* (a) (b)	Dieletric Constant** (c)	Solvent	Solubility* (a) (b)	Dieletric Constant** (c)
Water	N/A	81.1	n-Butyl acetate	0.5 (25°)	5
Formamide	Miscible	84	Di-isopropoxy-methane		
Formic acid	do	58.5	Dipropoxymethane		
Acetonitrile	do	38.8	Nitromethane		39
Methanol	do	31.2	n-Butyl bromide		
Acetic acid	do	6.3	isoPropyl ether	0.2	
Ethanol	do	25.8	n-Butyl butyrate		
isoPropanol	do	26	n-Propyl bromide	0.3	
Acetone	do	21.5	n-Butyl ether		
n-Propanol	do	22.2	Methylene Chloride	2	
Dioxane	do	3	Chloroform	1 (16°)	5.1
Dimethylformamide	do	3.15	Dichloroethane	0.6	
Tetrahydrofuran	do		Bromobenzene	0.05 (30°)	5.4
t-Butanol	do		Trichloroethane		
2-Butanol	12.5		Ethyl bromide	0.9	
Methyl ethyl ketone	35.3 (10°)	18	Benzene	0.08 (22°)	2.24
Cyclohexanone	2.4 (31°)	18.2	Propyl chloride	0.27	
Phenol	6.7 (16°)	9.7	Trichloroethylene	0.1	
n-Butanol	7.9	19.2	Toluene	0.05 (16°)	2.3
Cyclohexanol	5.7 (15°)	15	Tetrachloromethane	0.08	2.25
isoAmyl alcohol	2.7 (22°)		Carbon disulphide		2.6
n-Amyl alcohol	2.7 (22°)	16	Decalin		2.13
Benzyl alcohol	4 (17°)	13	Cyclopentane		
Ethyl acetate	8.6	6.1	Cyclohexane		
n-Hexanol	0.6		Hexane	0.01 (15°)	1.88
2-Collidine	3.5		Heptane	0.005(15°)	1.97
Ether	7.5	4.4	Paraffin oil		
Diethoxymethane					

* - ml of water in 100 ml of solvent
** - at room temperature

(a) Values from 'Handbook of Chemistry and Physics' (2).
(b) Unless noted, the given value represents the solubility at 20°C. Other temperatures are given in parenthesis.
(c) Values from 'Taschenbuch fur Chemiker und Physiker' (3).

The choice of solvent is not entirely a matter of guesswork. The carbon to oxygen (C/O) ratio of the analytes is some guide. If it is very low, between 1 and 2, a polar mobile phase is used. For values between 2 and 5, use solvents of intermediate polarity and over 5 non-polar solvents such as hydrocarbons. In principle, like is used with like, a more polar solvent mixture being used to separate more polar substances.

The R_f value is the ratio of the distance travelled by the spot divided by the distance travelled by the solvent front. If this value is too low using the solvent chosen, repeat with a more lipophilic one. If the R_f values are too low even with a very polar mixture such as n-butanol:acetic acid:water (4:1:5) (the Partridge mixture) a single phase system may be tried such as a 2.5% sodium chloride solution or aqueous solutions of acids, alcohols or ammonia. If this fails, an alternative technique must be used. If the R_f values are too high even with a non-polar solvent, separation may be attempted using a reversed-phase technique, or using a polar organic solvent as the stationary phase.

Ideally, the substances being separated should have R_f values in the range 0.15-0.85. Values of 0.00 and 1.00, though they can sometimes be used for separation, are not suitable for characterisation of any substance.

Addition of polar solvents (for example DMF, acetic acid, or ethanol) to non-polar solvents (for example chlorinated hydrocarbons or hydrocarbons) may have a number of beneficial effects, such as increasing the solubility of the substance in both phases and suppressing absorption on the paper. The result is rounder spots and higher R_f values.

Spots may be elongated (tailing or trailing) for a number of reasons including dissociation, poor solubility, absorption on the paper, and poor equilibration. Run a chromatogram with spots of different concentration. If the more concentrated spots tail worse, but the front edge has the same R_f for all concentrates, the tailing is due to poor solubility and a better solvent must be found. If the more concentrated spots tail worse and also have front edges or centres of higher R_f, the overall shape being that of a comet, the cause is either dissociation or absorption. Very slight dissociation and complete dissociation give rise to compact spots, it is only if it is partial that tailing occurs. This is a problem with substances such as colours. It is solved by either suppressing the dissociation, or making it complete. For example, the dissociation of acidic substances can be suppressed by chromatographing them in an acidic solvent (the usual choice) or can be made complete by use of an alkaline solvent. Alternatively, the paper may be impregnated with a buffer that will maintain a pH at which the dissociation is shifted in one direction or the other. Adsorption can be a problem in single-phase systems, but if tailing occurs in the usual two-phase system it is usually due to poor equilibration.

Application of Test Solutions

Sample and standard spots are applied to the paper at about 2 cm intervals and about 3 cm from the bottom edge for ascending development. It is convenient to draw a pencilled mark across the paper as the spots must all be in an exact straight line. Solutions are applied from capillary tubes or a micro-syringe. The size of the spot should be as small as possible, no more than 3 or 4 mm across. This is accomplished by applying the test solution in small volume increments and gently drying between applications. Drying can be done using warm air from a hair dryer or similar device.

Development and Chromatography

For ascending development, the spotted paper is suspended in a previously equilibrated tank, with the bottom edge of the paper immersed in the mobile phase to a depth of about 1 cm. The tank should be in an area free from draughts, direct sunlight and sources of heat. The tank may be lined with filter paper soaked in the mobile phase in order to hasten equilibration. The chromatogram is allowed to develop at least 10 cm (considerably more is necessary for some separations). Drying and redevelopment may result in improved separation. The paper is finally removed and dried. If the substances are heat-labile, leave to air dry. The stability of the substances may be affected by the solvent used; for example phenolic solvents increase the loss of peptides and amino-acids that may occur during drying.

Complete drying between multiple developments is not essential, but it is important in two-dimensional development, where remains of the first solvent may lead to unreproducible R_f values. If visualisation is likely to be affected by pH, any basic or acidic solvents used must be completely removed. Papers that have been treated (e.g. with buffers) should be air-dried, so that they do not buckle.

Descending development has been used for the separation of sugars and colours, among other groups. The paper is held in a trough by a heavy glass rod or other means, and the trough filled with mobile phase.

Two-dimensional chromatography is used for the separation of complex mixtures such as amino-acids. One spot only is placed in a corner of the paper and the chromatogram is developed. The paper is removed, dried, turned through 90° and developed with a different mobile phase. Standards must be on a separate paper. This is often the only way to separate closely related compounds.

Visualisation

The developed paper is sprayed with a suitable reagent, or dipped in a solution of it in a trough. Many of the reagents are toxic to a greater or lesser extent and the fineness of the spray increases the likelihood of inhalation, so the spraying should be done in a fume hood with an efficient exhaust system. The spray should be kept some distance from the paper so that larger drops are lost and only the finest reach the paper, giving a more even colour development. There are many types of glass spray bottles commercially available. It is advisable to use a commercial unit rather than attempting to construct one, as they generally deliver a more uniform spray.

Spots may also be visualised by exposing the paper to vapours such as ammonia or acetic acid as well as iodine. This may sometimes be a means of rescuing a paper that has been sprayed without complete removal of an acidic or basic solvent.

Pain (4) describes an improved method using iodine. The dry paper is sprayed on both sides with aluminium sulphate solution (20% w/v of the hydrate) and re-dried. It is then left in iodine vapour at least three hours and preferably overnight. Excess iodine is removed by leaving the paper suspended one hour, and then it is sprayed with 0.5% starch solution. Spots may appear as dark blue on a light blue background. Lipids, alkaloids, and amino-acids can be visualised in this way.

5.2 THIN-LAYER CHROMATOGRAPHY (TLC)

Many of the remarks under the section on paper chromatography also apply to thin-layer, although the separation principle involved is quite different. In thin-layer chromatography, the substances are absorbed to a greater or lesser extent on the solid layer and the principal mechanism of separation is elution from it by the mobile solvent. However, in reversed-phase TLC, the principle of separation is similar to that in paper chromatography.

As was done in the above section on paper chromatography, this section on TLC will discuss the materials used and their preparation, along with sample application and development.

Selection of TLC Layers and Supports

The most commonly used materials are silica gel, alumina, polyamide and cellulose powders but others are sometimes used, such as polycarbonate, magnesium silicate, etc. The layer must be supported on glass, aluminium sheet, plastic or other inert material. Aluminium or plastic are most convenient if the plates are purchased already prepared, as they can be cut by scissors and thus used more economically. Such plates usually give more satisfactory results than ones prepared in the laboratory and are therefore to be preferred. The letter G after the name of the substance indicates that calcium sulphate has been added to it as a binder, traditionally at the level of 13%. The letter H indicates that no binder has been added. the symbol F_{254} indicates that the thin layer fluoresces at 254 nm due to incorporation of a fluorescing agent.

Preparation of Glass Plates

Spreading devices are available commercially. The operating instructions must be followed. A slurry is prepared from water and the powder to be used (about 2 + 1 by weight for silica gel). (Alternatively the powder can be mixed with

an organic solvent). Silica gel slurries are best homogenized in a blender for a couple of minutes. If this is done, it may be necessary to increase the amount of water slightly to obtain a slurry of the right consistency. The glass plates, which must be on a perfectly flat surface such as a plate leveller, are fed into the spreader through a gate which has been set with a feeler gauge to the appropriate thickness. Glass sheets, 20 x 20 cm or 5 x 20 cm are normally used. 0.25 mm is a suitable layer thickness for routine applications. Anandaraman et al (5) describe a simply constructed glass spreader.

An application device can be constructed using zinc oxide plaster tape. Most brands of zinc oxide plaster are about 0.25 mm thick. Place five 20 x 20 cm plates on a perfectly flat surface butting against each other in a line and hold them in place by plaster tape along the two lengths and one end of the line, allowing the plaster to overlap the glass by about 0.5 cm. See illustration in Figure 5.2.

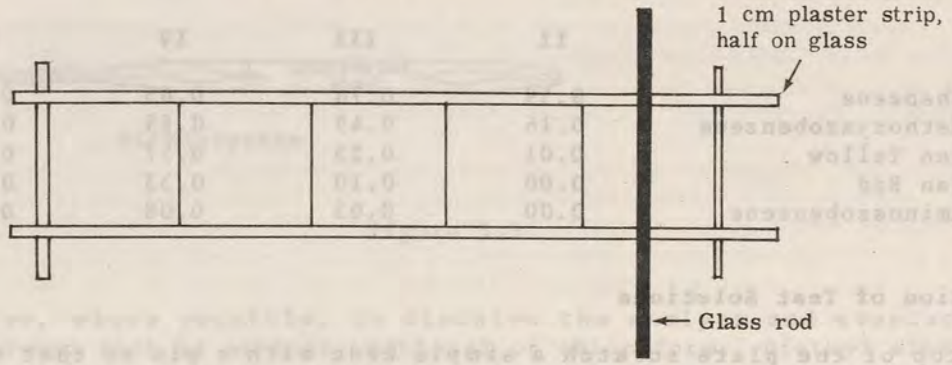


Figure 5.2

A slurry (e.g. 30 gm silica gel + 70 ml water is enough for five 20 x 20 cm plates) is poured onto the plates from a beaker in the left hand and a stout glass rod (about 30 mm long) is moved across the plates immediately behind the beaker. After a little practice the slurry can be poured on, and the rod moved along at the correct speed so that by a rapid, smooth action all the plates are evenly coated. Instead of using tape along the plate edges, cellotape or scotch tape may be wound around the glass rod at two points about 19 cm apart and the rod drawn along the plates just behind the slurry as the latter is poured. If this method is used, it is advisable to hold the plates in place in some other way.

Layers may also be supported on microscope slides. The dry clean slide is dipped into the slurry, air-dried and the layer on one side brushed off.

For some separations, it is necessary that the layer be impregnated with a buffer, a non-polar substance or some other aid to separation. For example, plates impregnated with silver nitrate (argentation chromatography) are useful in separating saturated fatty acids from unsaturated, as the latter form adducts with the silver nitrate and hence travel more slowly. Impregnation with a non-polar substance and subsequent use of a polar solvent for development is called reverse-phase chromatography. If the plate is being prepared in the laboratory, the substance with which the layer is to be impregnated is incorporated in the slurry instead of using pure solvent. The layer on commercial plates is usually firm enough so that they can be dipped into a solution of the substance in a tray and then redried or re-activated. Some care may be needed to ensure that impregnation occurs evenly.

Drying and Activation of Prepared Plates

Prepared plates must be air-dried until they can be moved without damaging the layer. Plates of silica gel and alumina are then activated by oven-heating and may be stored in a desiccator. Silica gel may be activated by heating 20 minutes at 110°C or at 100°C. Alumina is generally activated by heating at the same temperatures for varying periods. Always avoid over-heating.

The activity of alumina on a prepared plate may be determined as follows: Prepare a dye mixture of 30 mg of p-azobenzene and 20 mg each of p-aminoazobenzene, p-methoxyazobenzene, Sudan Red and Sudan Yellow dissolved in carbon tetrachloride and diluted to 50 ml with the same solvent, Add 0.02 ml of this solution to the plate and develop in carbon tetrachloride. Water must be rigidly excluded. The activity of the alumina is determined by comparison of the R_f values of the dyes with the values in the following table:

Dye	Grade of Alumina according to Brockmann and Schodder(6)			
	II	III	IV	V
Azobenzene	0.59	0.74	0.85	0.95
p-Methoxyazobenzene	0.16	0.49	0.65	0.89
Sudan Yellow	0.01	0.25	0.57	0.78
Sudan Red	0.00	0.10	0.33	0.56
p-Aminoazobenzene	0.00	0.03	0.08	0.19

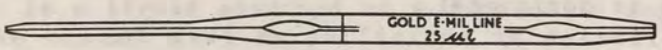
Application of Test Solutions

At the top of the plate scratch a simple code with a pin so that there is no confusion about the identity of the spots after visualisation. Solutions should be applied at 2 cm intervals and about 1.5 cm from the edge of the plate. Spot parallel to where the layer extends to the base edge. If the layer is firm enough, pricks may be made at 2 cm intervals in a straight line with the aid of a pin and a ruler. The solutions are then spotted in between the prick marks, judging by eye that the spots are in a straight line. It is preferable, and essential if the layers are loose, to use a transparent template with raised edges so that it stands over the plate, and having a row of holes on one side through which the solutions may be spotted. Do not spot samples too near the edge of the plate, as the R_f will be altered (the edge effect). It is because of this effect that the few millimetres of the layer on each of what will become the two vertical sides of the plate should be removed, if present. This is conveniently done by running along the edge a clean cork in which a right angle of the required length has been cut. Try to keep the spot less than 2 mm across. This is easily done in qualitative work, as very fine capillaries may be drawn and used. Tiny spots about 0.5 mm across give considerably better results than those from spots a few millimetres across, and extra care is worthwhile. If a semi-quantitative result is required, the volume applied must be measured with a micro-litre syringe such as the Hamilton type, an Agla microsyringe, or a graduated microcapillary (Oxford type or a microcap) (See Figure 5.3). Usually, one to five microlitres are applied but it may be possible to apply up to 50 microlitres without the layer breaking up. It is preferable to use a stronger solution rather than try to spot these larger amounts. The concentration of standards to be used depends on the sensitivity of the method of detection, but a microlitre or two of a 1% solution is commonly used. If the method of detection is fairly sensitive, a 0.1% solution may be better. The capillary containing the solution is touched against the layer so that liquid is absorbed without the surface being spoiled. It is more difficult to contain the size of the spot when measured volumes are being applied. A small plastic jet attached to the end of the syringe or capillary may help in placing a smaller volume on the plate at each application. The drying of the spot can be hastened by use of a hair-dryer or other source of hot air. The drop should not be allowed to dry as it leaves

the syringe, or the analyte might collect at the tip and not reach the layer. The syringe should be rinsed with a microlitre or two of solvent and this applied to the same spot, but if the substance has been deposited on the outside of the tip, rinsing with solvent may succeed only in leaving it behind in the solvent beaker. If glass plates are being used, it may be more satisfactory to keep the plate on a warm surface rather than use a blower.

Hamilton syringe

Agla microsyringe pipette



Micropipette

Figure 5.3

It is better, where possible, to dissolve the analyte and standards in a volatile solvent such as acetone, methanol or chloroform. Diethyl ether is too volatile for most purposes, water not quite volatile enough, so that greater care is required to restrict the size of the spots.

The R_f value of a substance will alter to some extent with concentration so even if only qualitative results are required it is important to add amounts of sample and standard as closely similar as possible. A satisfactory procedure is to apply several standards spots, some containing more and some less of the analyte than the sample spot. It is advisable to apply the least amount that gives a recognisable spot.

A number of devices are on the market that automate the spotting of the sample to a greater or lesser extent. They achieve a more regular application of the solutions than is possible manually and therefore better results, but are not essential for routine work. Improved results can be obtained by applying a line or a band of solution to the plate rather than a spot. This is difficult normally, but works very well with one of the mechanical applicators available commercially.

R_f values on TLC plates are not reproducible and constant as they depend upon factors such as temperature, fineness of absorbent, relative humidity, activity of the layer and so on. It is therefore convenient to use a 'marker' substance and calculate R_f values relative to it.

Development and Chromatography

The chromatogram is developed in a tank with a close-fitting lid and containing solvent to a depth of about 0.5 cm for a 20 x 20 or 5 x 20 cm plate. The solvent should be left in the tank for at least an hour before running the chromatogram. The walls of the tank may be lined with filter paper soaked in the developing solvent in order to bring the vapour to equilibrium more quickly. The tank should be placed in an area free from draft, having an even temperature and away from direct sunlight.

Small screw-cap jars, lipless beakers covered with watchglasses and similar containers may be used for plates cut from aluminium or plastic sheets, or for layers on microscope slides. The staining jars used in histology are also satisfactory for the latter.

The solvent is allowed to run up the plate to 10 cm above the starting line (for 20 cm high plates) or further if desired. The 10 cm line may be marked on the side of the plate with a pin before the run is started. Various modifications may be of use. For example, a single spot may be placed in the corner of a plate, which can be developed in one solvent, dried, turned through 90° and developed in another solvent (two-dimensional TLC). In this technique several plates have to be run if standards are included. In ordinary one-dimensional development a certain improvement is obtained if the chromatogram is run, dried, and then run again in the same solvent (multiple development). Alternatively, stepwise development may be carried out with different solvents. The chromatogram is run a few centimetres in the least polar solvent, removed, dried, and then run further in the solvent of next polarity and so on. The remarks on polarity, choice of solvent and improvement of separation under "paper chromatography" also apply to separations on thin layers. If loose layers without binder are used these are so delicate that development must be carried out with the plate close to horizontal.

Overspotting

If sample and standard appear to be identical, a fresh plate should be prepared, including sample, standard and sample with standard spotting on top of it. If the two travel together, and this can be repeated with two further solvents, it may be assumed that standard and sample are identical. Exceptions are rare, e.g. the colours Ponceau SX and Allura Red.

Visualisation

After removing the chromatogram from the tank and drying, it should be examined under ultraviolet light, both shorter wave (254 nm) and longer wave (365 nm). If a fluorescor has been used that fluoresces under the shorter wave, many substances show up as dark spots where they have quenched the fluorescence. These spots may be lightly circumscribed with a pin to mark their position before spraying. After spraying (which uses the same devices as mentioned under "Paper chromatography") the plate may require heating before spots appear. Sometimes the plate can be sprayed with a succession of reagents, as with Korbelak's method for the detection of artificial sweeteners.

Useful general visualising agents include iodine. A few crystals can be scattered on the bottom of a large desiccator and the plate left in the vapour for half an hour or so (depending on ambient temperature). Most organic compounds show up. The charring action of concentrated sulphuric acid may also be utilised. The plate is sprayed with the concentrated acid and heated to 110-140°C. Inclusion of 0.5% vanillin or salicylaldehyde gives colours in the cold with many substances, and other colours on heating. It is less hazardous to spray with acid ammonium sulphate solution (50%) and heat the plate (for example on a hotplate in a fume cupboard). The ammonia is driven off and sulphuric acid remains. Phosphoric acid can also be used in the same way. Another useful general reagent is weak potassium permanganate solution, either acid or alkaline.

5.3 GAS-LIQUID CHROMATOGRAPHY (GLC)

A gas-liquid chromatograph is shown schematically in Figure 5.4.

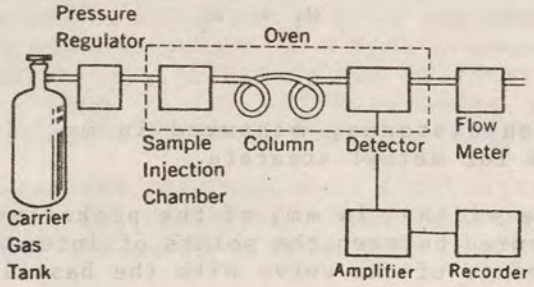


Figure 5.4

A solution of the sample is injected on to the column and swept through it by an inert carrier gas (the mobile phase). For packed columns the stationary phase is a liquid adsorbed on a fine solid support material. The temperature of the column must be sufficient to volatilise the sample and allow it to partition between the mobile and stationary phases. The greater the solubility of a substance in the liquid phase the slower it travels. In a mixture, the various components will therefore separate depending on their interaction with the liquid stationary phase. When the compounds leave the column, they are sensed by the detector and are recorded as a trace on a moving graph. In the case of a capillary column, the liquid forms a thin film on the walls of a very narrow tube and no solid support material is required.

Gas chromatographs are expensive and it is important that the analytical staff be well trained in their use. This training is often offered by the manufacturer. This should be a consideration when a new instrument is being purchased.

There are several potential problems which become important when operating gas chromatographs:

1. Voltage supply must be stable. Many GLC units have built-in voltage stabilizers. If not, a stabilizer (usually a transformer to smooth out surges) must be installed between the instrument and the voltage source.

2. The carrier gas must be pure enough for routine work. If the purity is unknown, it is best to use a molecular sieve between the gas tank and the instrument. The instrument manufacturer can usually recommend a correct sieve. This only applies to gases used as a carrier. Other gases such as hydrogen used in a flame ionization detector, do not need the same purity requirement.

3. It is generally best to use glass for column material as metal columns often catalyze decomposition reactions while the sample is going through the column.

The efficiency of a GLC column is based upon the number of theoretical plates it contains. The following example calculation of the theoretical plates uses the separation of stearate and oleate:

Number of theoretical plates, n, (or the efficiency):

$$n = 16 \left[\frac{dR}{W_1}\right]^2$$

The resolution, R, is given by, (see Figure 5.5):

$$R = \frac{2D}{W_1 + W_2}$$

where:

dR is the retention distance, measured in mm, from the start to the maximum of the peak for methyl stearate.

W_1, and W_2, are the widths, in mm, of the peaks for methyl stearate and methyl oleate, measured between the points of intersection of the tangents at the inflexion points of the curve with the base line.

D is the distance between the respective peak maxima for methyl stearate and oleate.

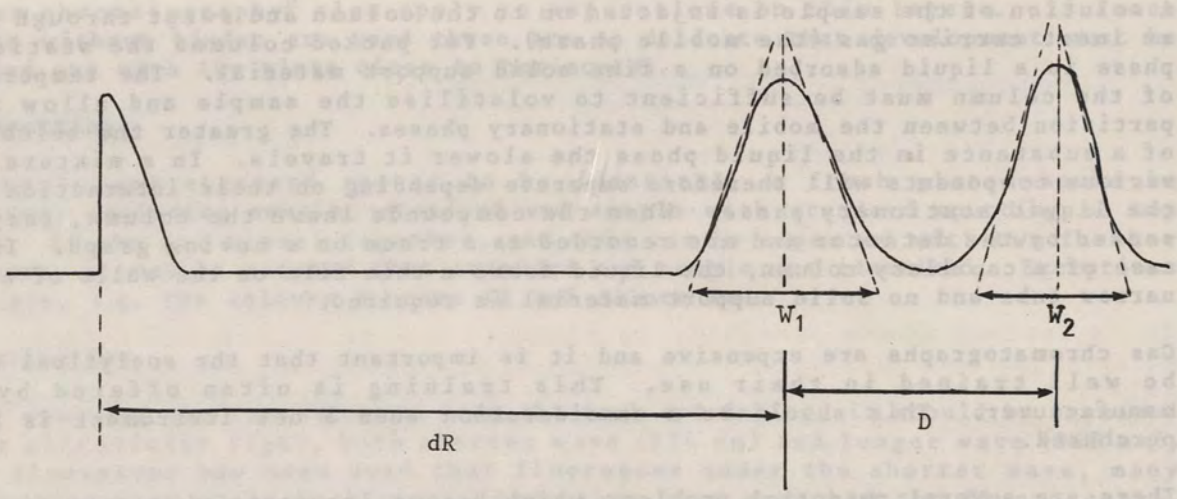


Figure 5.5

Some additional precautions to keep in mind while operating a gas chromatograph:

1. Always have the injection port at least 10°C higher than the column temperature. This assures the sample and solvent will be properly volatilized.

2. Change injection septa frequently as they are prone to leak because of age and/or frequent piercing.

3. Check the carrier gas flow rate at least once per day.

4. Check for gas leaks (with soap solution) each time gas connections are re-fitted.

5. Do not tighten brass swage-lock type fittings excessively as they may be damaged.

The best routine detector to use in food analysis is the FID or flame ionization detector. This detector "sees" any compound which will ignite in the FID flame. This, of course, includes all organic matter, so that the major drawback of the FID is its non-selectivity. Selective detectors are useful as they ignore everything except what they are designed to detect. As example, a selective nitrogen detector will "see" only nitrogen containing compounds and will be blind to all others. This means that many pre-separations or purifications do not have to be done when a selective detector is used.

5.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Liquid chromatography is a very useful technique to separate compounds which are not easily separable by other means. Examples include ionic substances, large molecules and materials which are readily degraded by heat. However, by its very nature, liquid chromatography is a slow process as low flow rates give the best separation efficiency. This is due in great part to the very slow diffusion rate of a solute in the solution being chromatographed. This diffusion rate is more than 50 times slower in a liquid than in a gas.

Modern HPLC instruments have overcome this difficulty by the development of columns containing very small particles. This increases the rate of diffusion dramatically and permits separations nearly as quickly as with gas chromatography.

A basic isocratic HPLC unit can be considered to consist of:

1. An injection loop.
2. A high pressure pulse-less pump.
3. A column with appropriate packing.
4. A detector.

In gas chromatography, the liquid sample is injected directly into the carrier gas stream. This direct injection is impractical with HPLC because of the tremendously high pressure the carrier liquid is under. Instead, a loop is used which has a reservoir where the sample is injected. This is then introduced into the carrier liquid stream without loss of pressure.

HPLC pumps must be capable of operation up to at least 6000 psi and deliver a liquid flow without surges or pulses. The usual HPLC pump is mechanical and delivers a constant flow rate, with the pressure dependent on the resistance of the liquid through the column. There is also the gas displacement type HPLC pump which delivers constant pressure, with the flow rate depending on resistance. Either are usable.

Small particle size for column packings are the key to rapid HPLC separations. An example is 10 micron size porous silica gel particles. Even smaller particles can be used, but these are often bonded on 30-50 micron glass beads to form a large surface area without restricting the column flow. Such coated beads are called pellicular beads and are especially useful if it is desired to coat them further with a thin layer of a liquid stationary phase. See Gray (7) for a discussion of columns used in food analysis.

The two most useful HPLC detectors, in food analysis, are the ultraviolet and the differential refractometer. The ultraviolet detector consists of a flow-through micro cell in a UV spectrophotometer. Commercial UV detectors range from one fixed wavelength (usually 254 mm) to variable wavelength over the whole UV spectrum. As most carrier liquids do not absorb in the UV, these detectors are very useful for compounds having some UV absorption. Some spectrophotometric detectors include the visible wavelengths as well as the UV, thus permitting HPLC analyses of food colours among others.

The differential refractometer monitors the difference in refractive index between the pure carrier liquid and the carrier plus sample.

5.5 SPECTROPHOTOMETRY

When light is passed through a liquid some of the radiation may be absorbed. The amount absorbed varies with wavelength and with the identity and concentration of the solute. This is the basis for all absorption spectrophotometry. As long as the light intensity is strong enough to pass

through without all being absorbed, its actual intensity is immaterial. It is the difference in intensity between incident and transmitted light that is measured. MacLeod (8) pointed out that absorptiometry is not particularly sensitive as it only measures differences in light intensity. However, the sensitivity achieved is adequate for most needs in regulatory food analysis.

The amount of absorbance of radiation depends upon the number of molecules in the light path and hence depends on the depth of the measuring cell and the concentration of the solution. This is an expression of the Beer-Lambert Law, which may be put in formula form as:

$$A = \log \frac{I}{E} = \epsilon ct$$

where:
- A = absorbance
- I = intensity of incident light
- E = intensity of light emerging from the solution
- ϵ = molar absorptivity
- c = concentration in g-mol/litre
- t = thickness in cm.

The scale on the spectrophotometer may also give readings as transmittance (T):

$$\%T = \frac{100\ E}{I}$$

The molar absorptivity (ϵ) is equal to the absorbance of a solution containing 1 g-mol/litre in a cell of 1 cm thickness. The absorptivity of a substance is the absorbance of a solution containing 1 g/litre in a cell of 1 cm thickness.

From the formula $A = \epsilon ct$ it is seen that for an absorbing species in a cell of given thickness, a plot of A against c is a straight line. For any particular substance this may be true only over a certain narrow range. It is preferable to use only the straight part of the calibration curve whenever possible.

A classic example of the phenomena of absorption is provided by benzene when examined in the ultraviolet wavelengths. Benzene is transparent in the visible, but has a very characteristic absorption spectrum in the UV (see Figure 5.6).

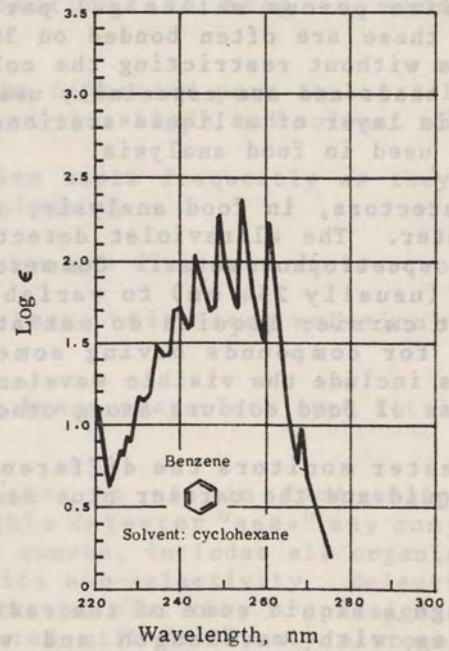


Figure 5.6

Absorption spectrophotometry (absorptiometry) is done in two main wavelength regions. These are, ultraviolet (approximately 200 nm to 350 nm) and visible (approximately 350 nm to 700 nm). Spectrophotometers are either single or double beam. In a single-beam instrument, the sample and reference (or blank) solutions are read alternately and the differing absorption values subtracted from each other. A double-beam instrument does this automatically by splitting the light from the monochromator between the sample and blank, so that the difference in absorption is subtracted electronically. This can be illustrated by the schematic diagram in Figure 5.7.

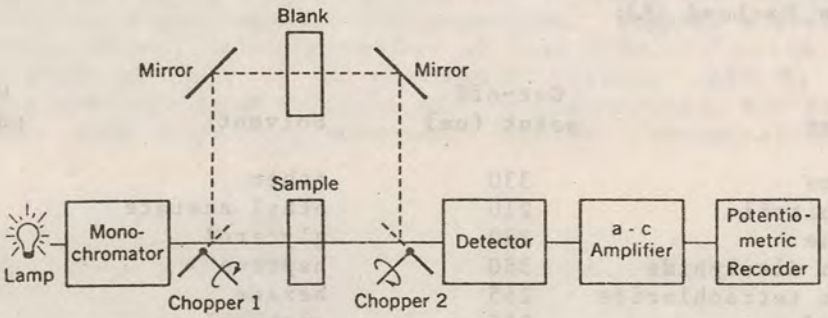


Figure 5.7

One of the key parts of a spectrophotometer is the monochromator. It takes the source light, divides it into different wavelengths using a prism or grating, and focuses light of a specific wavelength on the sample cell. This is shown in Figure 5.8.

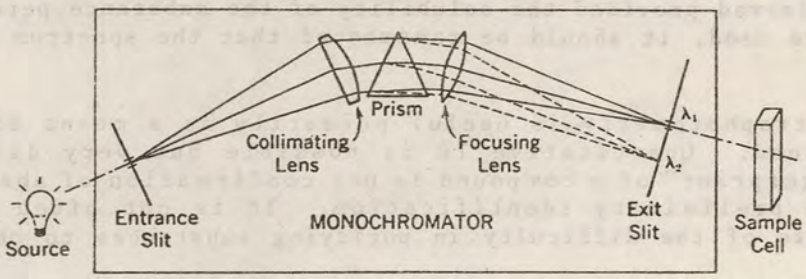


Figure 5.8

In scanning spectrophotometers, the prism or grating is rotated to give a continuous spectrum of wavelengths through the sample cell.

The absorption cell (also called cuvette) must be kept scrupulously clean. Optical surfaces should not be touched, as oil smudges are difficult to remove. Purity of solvents used for samples and for cleaning is important for the protection of the cells; only distilled water or spectrophotometric grade solvents should be used. As soon as possible after use, cells should be rinsed and soaked in distilled water. For cleaning, use a solution of mild detergent containing no lanoline, oils or insoluble matter. Ultrasonic cleaning equipment can be used to advantage in maintaining cell cleanliness and is especially helpful in removing materials which have been deposited on cell walls. Avoid prolonged contact with alkalies or hot, concentrated acids. Do not use abrasives or other agents which might mar the polished surfaces. A brush or other device capable of scratching cells should never be used. Avoid blowing cells with air to dry them; it is better to speed evaporation by means of suction. Cells should not be cleaned in chromic acid. Rinsing in acetone or alcohol often aids drying.

Glass cells are satisfactory down to about 350 nm. Below this they are opaque and silica cells must be used. Small differences will be found between cells. Even "matched pairs" may be exactly matched only over two or three short wavelength ranges. When using a double-beam instrument, it is useful to check the matching of the available cells at the wavelengths of interest. If there is a difference, make sure the sample cell has a positive absorption when compared to the blank or reference. Record this value and subtract it later.

All solvents have a lower wavelength limit or "cut-off" where they become opaque to light. Some limits are given below for common solvents. The table is taken from MacLeod (8):

Solvent	Cut-off point (nm)	Solvent	Cut-off point (nm)
acetone	330	ether	220
acetonitrile	210	ethyl acetate	260
benzene	280	glycerol	220
carbon disulphide	380	heptane	210
carbon tetrachloride	265	hexane	210
chloroform	245	methanol	210
cyclohexane	210	2-methylbutane	210
cyclopentane	210	methylcyclohexane	210
1,2-dichloroethane	230	nitromethane	380
dichloromethane	235	pentane	210
N,N-dimethylformamide	270	toluene	285
dioxane	220	m-xylene	290

Substances tend to give more detailed spectra in less polar solvents, which are thus to be preferred provided the solubility of the substance permits this. If water has to be used, it should be remembered that the spectrum may vary with pH.

Infrared spectrophotometry is useful primarily as a means to identify an organic compound. Quantitative IR is possible but very difficult. The infrared "fingerprint" of a compound is not confirmation of absolute purity, but is a good preliminary identification. It is not often used in food analysis because of the difficulty in purifying substances to obtain matching IR spectra.

Atomic absorption spectroscopy depends on the absorption by the atoms in the flame, of light of a specific wavelength emitted by a lamp or other source, of the same element as that being determined. Thus interference is generally rare. One type of interference is caused by absorption by molecules and other non-atomic species, absorbing continuously over a range of wavelength. This is usually only of practical importance below about 250 nm near the limit of detection of the element and in solutions containing high concentrations of salts. The difficulty is resolved by use of a deuterium or hydrogen background corrector, which measures the non-atomic absorption and subtracts it from the total signal.

Satisfactory results are obtained only if the sample solution is properly prepared, and freshly-prepared standards are treated in the same way. The viscosity and surface tension of the solution aspirated affects the rate of uptake through the nebulizer and hence the signal. The standards must be in a carrier liquid with the same physical properties. Dilute solutions of standards quickly deteriorate and should be prepared fresh each day from concentrates containing at least 1000 mg/l unless it has been established that the dilute standards are stable. For example, Moody et al (9) have shown that addition of about 1 ppm of gold tetrachloride stabilises very dilute mercury solutions in 0.5M HNO_3 in glass. The standard solution should be aspirated

after every half-dozen or so of samples to check that the instrument response has not changed. If the element being determined has been extracted into solvent, standards must be treated in the same way.

Matrix interferences, due to unavoidable differences in viscosity, surface tension or other properties between test solution and standard, must be overcome by use of the method of additions. If matrix interference is absent, results by direct aspiration and by the method of additions will be identical. The method involves adding known amounts of standard to aliquots of test solution and plotting signal against concentration. Suppose a test solution is thought to contain about 3 micrograms/ml of lead (Pb). Pipette 5 ml of this solution into each of four 10 ml graduated flasks. Add 0, 2, 3, 4 ml respectively of a standard lead solution of 5 micrograms/ml. and dilute all the flasks to 10 ml. Make a plot of absorbance against concentration (see Figure 5.9).

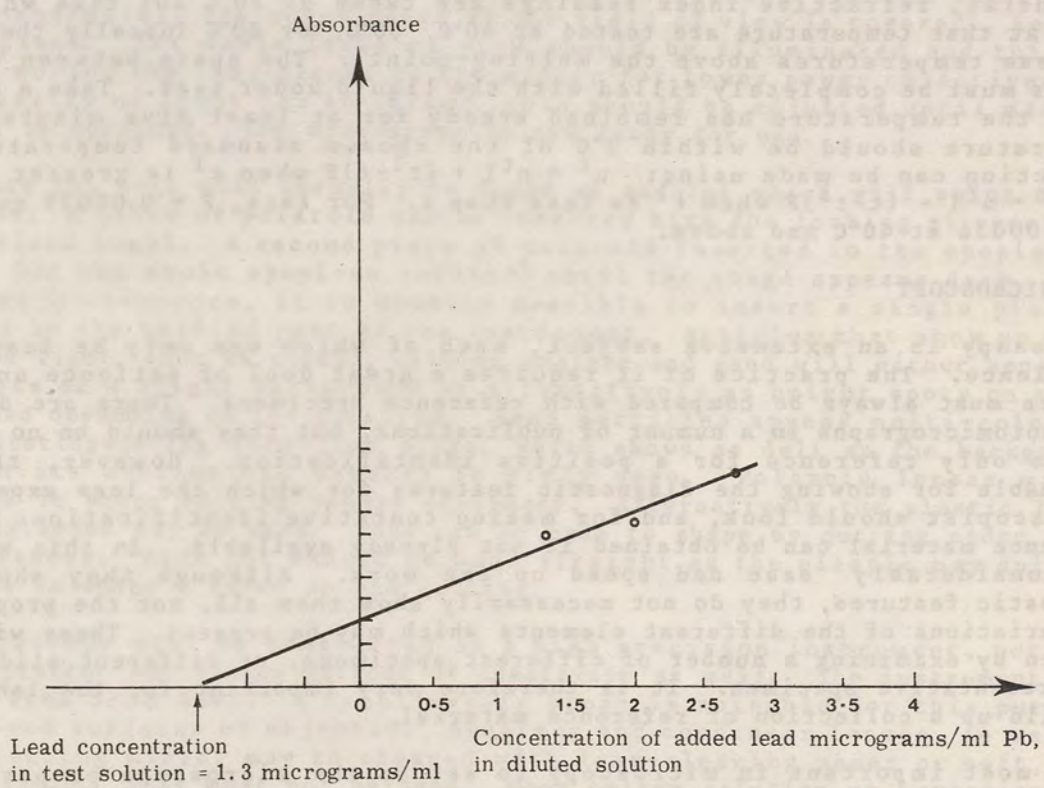


Figure 5.9

The concentration of lead in the diluted test solution is read from the negative abscissa as 1.3 micrograms/ml. Since this was derived from 5 ml of test solution diluted to 10 ml. the concentration in the original test solution was 2.6 micrograms Pb/ml. The amounts of added standard solution should be judged so that the calibration curve is at an angle fairly close to 45°, in order to maintain precision. Precision is also reduced by the small volumes pipetted, but this is often unavoidable in practice and should be alleviated by use of Grade A pipettes. There are other interferences, such as ionisation or formation of thermally stable chemical complexes, but these will not be covered. Most standard texts on atomic absorption spectroscopy include such information.

5.6 REFRACTOMETRY

The refractive index (n) of a medium is the ratio of the speed of light in vacuo to its speed in the medium. It is measured by the ratio of the size of the angle of incidence to the size of the angle of refraction where a ray of light passes from air into the liquid. Unless otherwise specified, the refractive index is reported for the mean wave-length of the D lines of sodium. For most refractometers in common use, the observation is made in white light but is corrected to sodium light by a compensating device in the instrument.

The refractometer is most useful for the examination of oils and of sugar solutions (e.g. as soluble solids in fruit juice preparations). It is important to keep the prisms scrupulously clean. The scale should be checked occasionally against water and alpha-bromonaphthalene or alpha-chloronaphthalene. The refractive index of liquids changes quite appreciably with temperature (and wavelength of the impinging light) and this must be borne in mind when checking the scale or testing samples.

In general, refractive index readings are taken at 20°C but fats which are solid at that temperature are tested at 40°C, 60°C, or 80°C (usually the lowest of these temperatures above the melting-point). The space between the two prisms must be completely filled with the liquid under test. Take a reading after the temperature has remained steady for at least five minutes. The temperature should be within 2°C of the chosen standard temperature. A correction can be made using: $n^t = n^t1 + (t^1-t)F$ when t^1 is greater than t, and $n^t = n^t1 - (t-t^1)F$ when t^1 is less than t. For fats, F = 0.00035 near 20°C and 0.00036 at 40°C and above.

5.7 MICROSCOPY

Microscopy is an extensive subject, much of which can only be learned by experience. The practice of it requires a great deal of patience and care. Samples must always be compared with reference specimens. There are diagrams and photomicrographs in a number of publications, but they should on no account be the only reference for a positive identification. However, they are invaluable for showing the diagnostic features for which the less experienced microscopist should look, and for making tentative identifications so that reference material can be obtained if not already available. In this way they may considerably ease and speed up the work. Although they show some diagnostic features, they do not necessarily show them all, nor the proportions and variations of the different elements which may be present. These will only be seen by examining a number of different specimens, or different slides from a representative specimen. It is therefore very important for the laboratory to build up a collection of reference material.

It is most important in microscopy to set up the microscope properly. It should be placed on a bench or table low enough to enable the observer to be seated and look comfortably down the microscope without having to tilt it. This is less of a problem with modern microscopes in which the tube is usually at an angle of 45° to the stage. There must be a bright source of light with its own condenser so that the beam can pass either directly, as with most modern microscopes, or by reflection from a mirror, through the microscope condenser to the object stage.

Best results will be obtained if the light and various parts of the microscope are aligned and adjusted for critical illumination. First of all, check that the Abbe condenser is centrally placed. To do this, make a small ink dot on the centre of the top lens of the Abbe combination and focus the dot with a low-power objective. Bring the dot to the centre of the field by adjusting the centering screws of the substage. Clean off the ink dot. Place a slide with a small object on the stage and focus a low-power objective on the object. If the lamp has a diffuser, this should be removed, or a sharp object such as a needle placed in front of it so that the needle or the lamp filament is seen through the microscope, which is focussed on one or the other by vertical

movement of the substage. After focussing replace the diffuser. If none is available, the substage may be moved until the lamp filament is just out of focus so that the filament image does not interfere with the view of the object. On many modern microscopes, the lamp is in the base of the instrument and is unlikely to be out of alignment, but if the lamp is separate and the stage lit via a mirror, the alignment must be checked. To do this, remove the objective and eyepiece lenses, place a dark glass somewhere between the lamp and the eye, and adjust the mirror of the microscope, the lamp and the lamp condenser so that the latter appears through the tube of the microscope as a clear round evenly-illuminated circle. It is important that the objective is filled with an even light. Provided the lamp and microscope remain in the same position, the lamp condenser need be focussed and aligned only when set up initially. Removal of lenses should be done only if necessary, as dust may collect in parts of the optical system, obscuring the image, and be difficult to remove. Once the various parts of the instrument have been aligned and the light focussed, it only remains to adjust the iris. After focussing the object, remove the eyepiece, close the iris to some extent and adjust it so that it becomes centrally placed if it is not so already. Adjust the iris so that approximately the outer third of the field of view is covered. Replace the eyepiece. The whole field of view should be illuminated and this may require moving the lamp closer to the mirror for lower power objectives. If the intensity of light can be varied, this should be adjusted until maximum contrast is obtained. The microscope is now ready for use.

Underneath the iris will normally be found an annulus which will swing out on its pivot. A piece of polaroid can be inserted here for looking at specimens by polarised light. A second piece of polaroid inserted in the eyepiece is rotated (or the whole eyepiece rotated) until the stage appears dark. With binocular microscopes, it is usually possible to insert a single piece of polaroid in the unified part of the instrument. Articles that show up under polarised light, such as starch, calcium oxalate and sand will either appear as dark spots on a bright background or alternatively as bright spots on a dark background depending on the rotation. Sand particles appear multi-coloured, easily distinguishing them from glass, which shows as dull as the background, although its outline can usually be dimly seen. Polaroid lenses may be purchased in shops selling camera equipment. Alternatively the plastic lenses from sunglasses may be used. They can be made to shape by cutting under water with scissors. Each cut should be made straight as the plastic may split if one tries to shape a curve at one cutting.

The microscope must be considered as a high precision instrument, not only during service, but during periods of inactivity as well. The instrument must be kept free from dust. A camel's-hair brush is suitable for this purpose. The exposed surfaces of objective, eyepiece and condenser lenses, as well as the reflecting mirror may be cleaned with lens-cleaning paper or soft, good quality linen. Silk must not be used. When balsam solution or immersion oils contaminate lens surfaces, a soft cloth or lens paper moistened with xylene should be used for cleaning. Alcohol must not be used for this purpose as it has a marked solvent action upon lens-mounting cements. Constant cleaning with xylene will have the same results. Objectives must never be taken apart in order to clean inside lens surfaces. They should never need it. Eyepieces on occasion do need unscrewing for cleaning purposes, but great care is necessary and the fingers should not touch the lens surface as the resultant greasy film deposited is very difficult to remove.

When the microscope is not in use, the low-power objective should be left turned around towards the stage and the low-power eyepiece in position. Other lenses should be put away in their containers. Damage to objectives is far commoner than it need be, the chief cause being the racking down of the tube sharply on to the slide. In the case of non-immersion lenses the following procedure must be adopted: Before looking into the eyepiece, the objectives should be lowered carefully until the front lens almost touches the coverglass

or object under examination, then while carefully looking into the eyepiece for the appearance of an image, the tube is slowly elevated by means of the coarse adjustment. Final focussing is then obtained by means of the fine adjustment. The diversity of structures found among plant cells is an aid in the identification of the plants from which foods are derived. In the case of powdered foods such as ground spices, microscopical examination not only assists in identifying the powder, but also may enable the analyst to detect the presence of adulterants. For example, sawdust (a common adulterant) consists largely of supportive and conducting tissue (vessels, tracheids, fibres and sieve-tubes) and these elements are likely to be easily distinguishable under the microscope from the elements characteristic of most spices. When examining food microscopically, it is important to look at several slides in order to assess the relative proportions of the different elements present, as well as search for diagnostic features.

It is not possible to deal adequately with the microscopy of foods in several pages. A few simple diagrams and a short glossary are given here so that the basic terms and the crude morphology may be understood. Those wishing to have a better understanding of plant anatomy are referred to Esau's, "Plant Anatomy"(9) and "Anatomy of Seed Plants"(10). There follows a brief description of the structure of the leaf, seed and fruit. Although these are the parts of plants most commonly used as food that require microscopical examination, other parts are also used, for example bark (cimmamon, cassia), roots (ginger, turmeric), flower buds (cloves), stigmata and upper parts of the styles (saffron), etc.

Figure 5.10 illustrates the transverse section through the midrib of a tea leaf (Camellia sinensis). Figure 5.11 is the same transverse section, showing elements in the cortex. Both illustrations are from the "Textbook of Pharmacognosy" by Wallis(12).

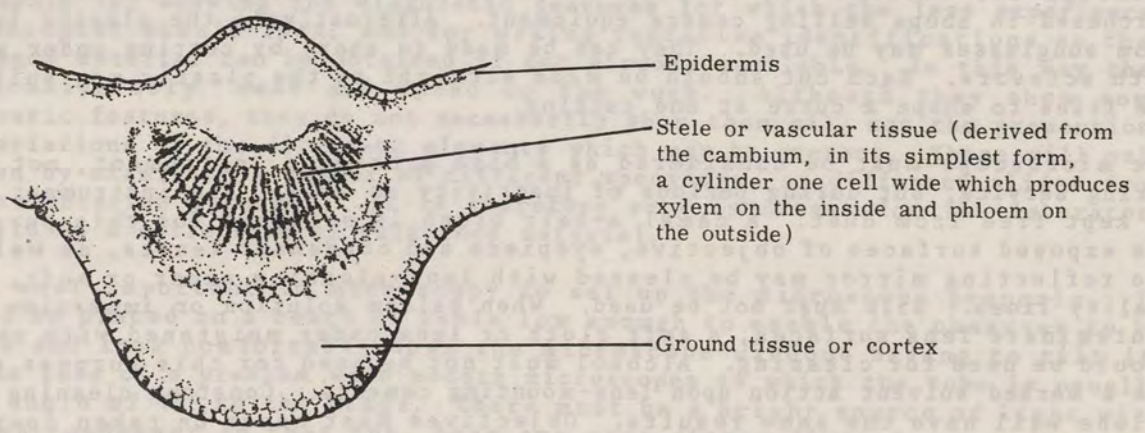


Figure 5.10

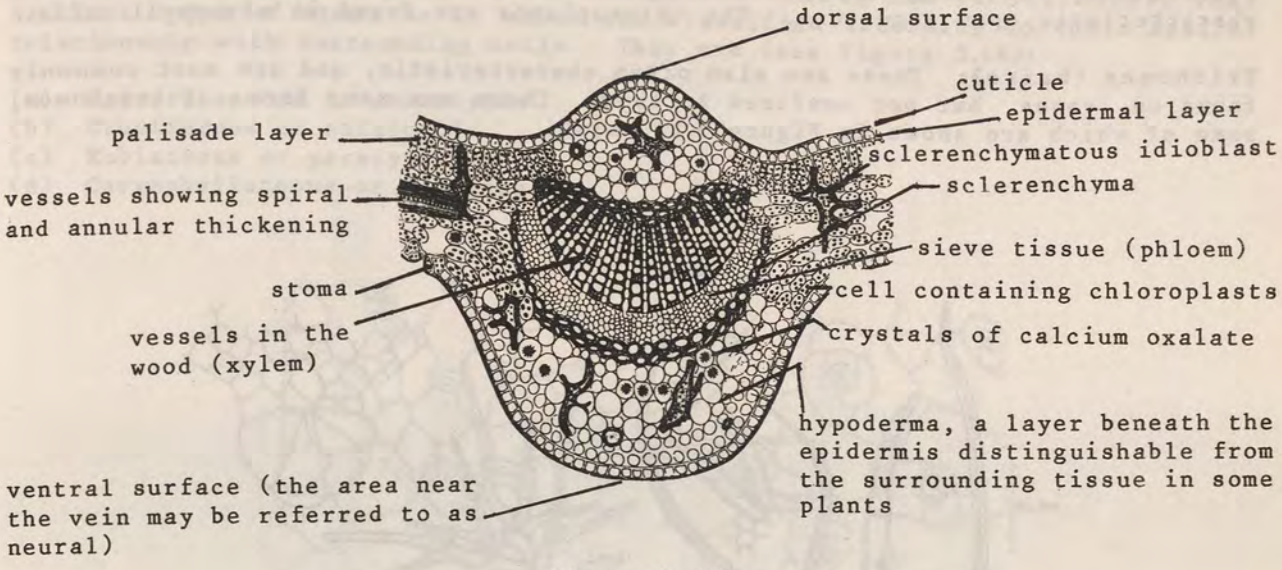


Figure 5.11

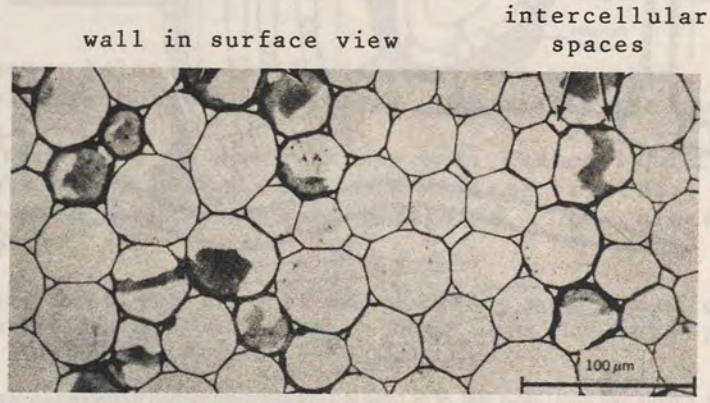
The following is a glossary of terms used in plant microscopy:

Dorsal and Ventral: For those leaves with a distinct upper and lower surface, they may be referred to as dorsal and ventral respectively.

Epidermis: This is usually composed of a single layer of cells.

Cuticle: Composed of cutin, a wax on the outer surface of the epidermal cells making them more or less impervious to water. The cuticle is prominent on some leaves as a brown, somewhat crinkled layer.

Parenchyma: This is tissue from any part of the plant composed of cells which have not differentiated to any special shape or composition and are therefore approximately rectangular in cross-section and are generally thin-walled. Such cells may be described as isodiametric, i.e. all diagonals are of equal length. (See Figure 5.12).



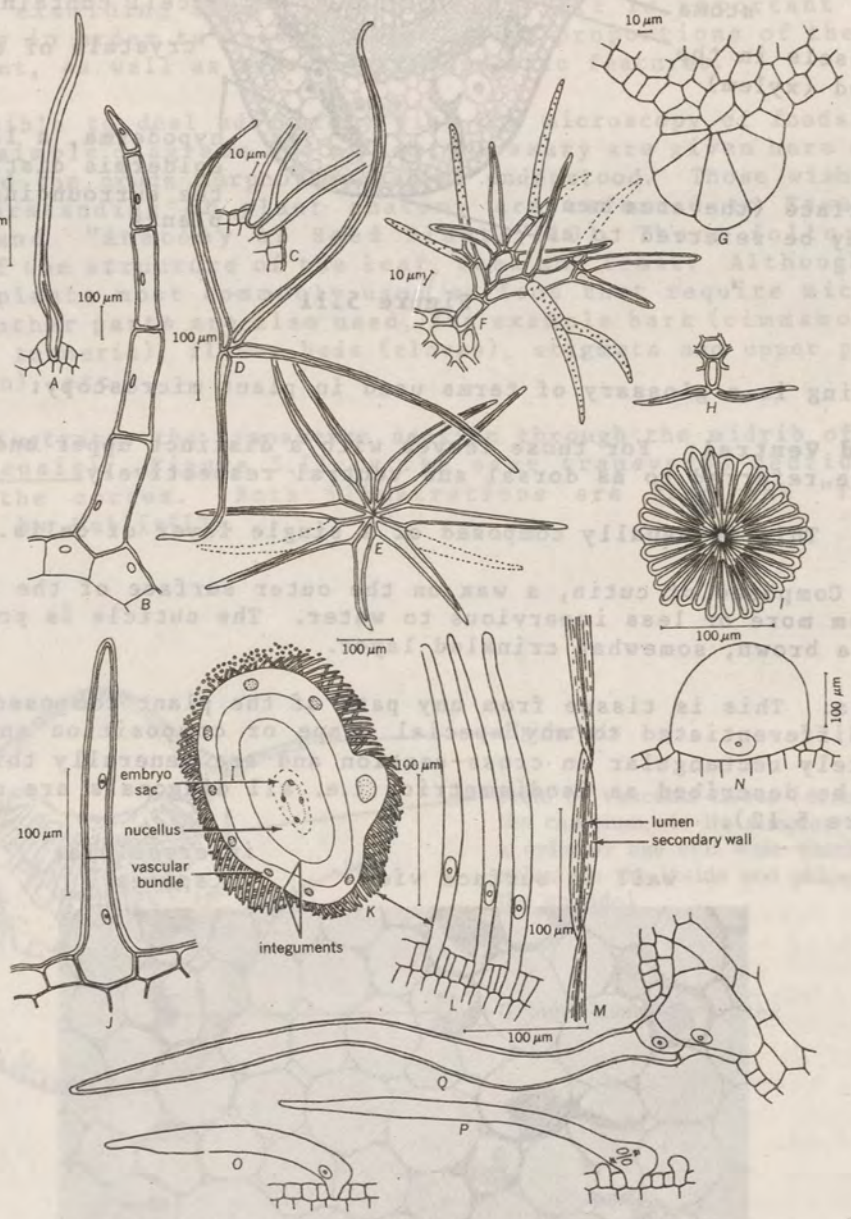
a thin-walled type of parenchyma, with regularly shaped cells and schizogenous intercellular spaces, from petiole of celery

Figure 5.12

Palisade layer: This is one or two cells thick and occurs under one or both epidermises. The cells are typically tabular (polygonal prisms), with no intercellular spaces.

Mesophyll: This includes all parenchymatous tissue between the two epidermises, including the palisade layer and the spongy mesophyll which has a characteristic open structure. The chloroplasts are found in mesophyll cells.

Trichomes (hairs): These are also often characteristic, and are most commonly found on leaves, but not confined to them. There are many forms of trichomes, some of which are shown in Figure 5.13 below.

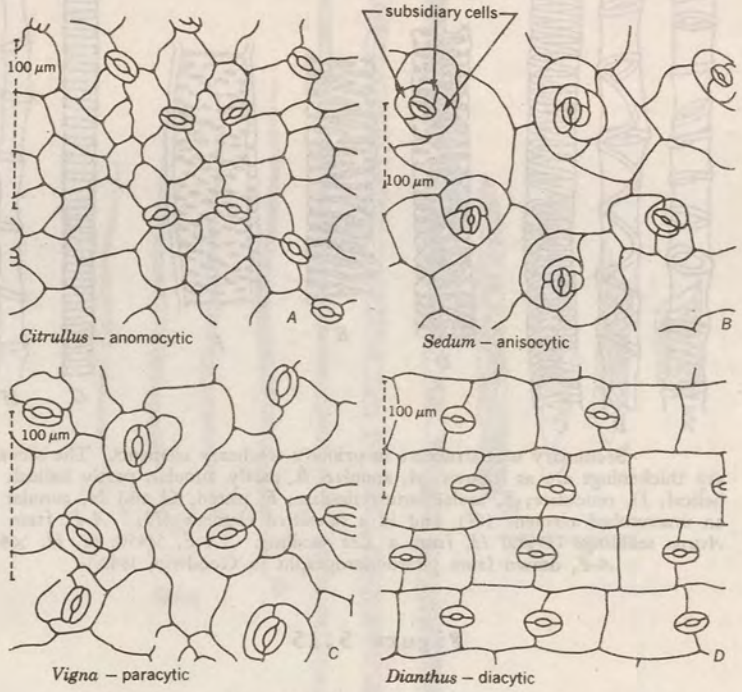


Trichomes. *A*, simple hair from *Cistus* leaf. At its base is a compartment formed by deposition of a siliceous wall. *B*, uniseriate hair from *Saintpaulia* leaf. *C, D*, tufted hair from leaf of cotton (*Gossypium*). E, stellate hair from leaf of alkali mallow (*Sida*). *F*, dendroid hair from lavender leaf (*Lavandula*). *G*, short multicellular hair from leaf of potato (*Solanum*). *H, I*, peltate scale from leaf of olive (*Olea*). *J*, bicellular hair from stem of *Pelargonium*. *K–M*, cotton (*Gossypium*). Epidermal hairs from seed (*K*) in young stage (*L*) and mature, with secondary walls (*M*). *N*, water vesicle of *Mesembryanthemum*. *O–Q*, hairs in three stages of development from leaf of soybean (*Glycine*).

Figure 5.13

Stomata: These are special cells in the epidermal layer through which the plant regulates exchange of water and gases. Among the dicotyledons, four distinct types of stoma occur which are classified according to their spatial relationship with surrounding cells. They are (see Figure 5.14):

(a) Ranunculaceous or anomocytic
(b) Cruciferous or anisocytic
(c) Rubiaceous or paracytic
(d) Caryophyllaceous or diacytic



Epidermis in surface views illustrating patterns formed by guard cells and neighboring cells.

Figure 5.14

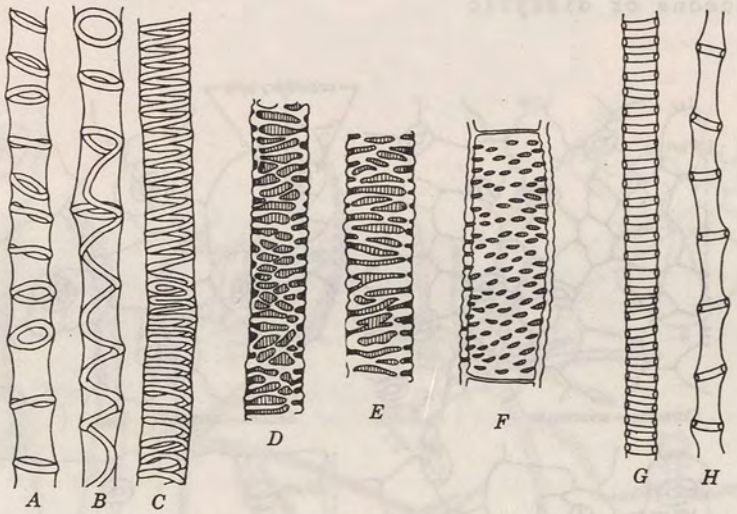
Stomata may be above, below or level with surrounding cells. They occur on the aerial parts of plants, but more on leaves, especially the undersurface.

Idioblast: The term used to describe any cell radically different from those near it, e.g. cells which contain calcium oxalate crystals. Calcium oxalate crystals show up well between crossed polaroids. They are commonly found in many parts of plants.

Prosenchyma: This name may be applied to tissue made up of cells in which the length greatly exceeds the breadth. They usually have pointed ends and thickened walls.

Vessels: A type of cell through which water is conducted in the plant. They are found as long wide tubes with thickened walls and no living contents, the end-walls usually partly or totally broken down, perforated septa sometimes occurring at intervals, often at an angle to the tube walls. Helically thickened vessels are often prominent at the extreme ends of the veins in leaf tissue. Water is conducted through the vessels in the living plant. Vessels occur in the xylem.

Sieve tubes: These are also long, wide tubes, but the walls are not thickened. The contents may still be alive, although restricted to near the walls, and the cells are separated by sieve-plates. Sieve-tubes have alongside them companion cells with dense granular contents. Nutrients are transported through sieve-tubes in the living plant. Sieve-tubes and companion cells occur in the phloem (see Figure 5.15).



Secondary wall structure in primary tracheary elements. The secondary thickenings are as follows: *A*, annular; *B*, partly annular, partly helical; *C*, helical; *D*, reticulate; *E*, scalariform-reticulate; *F*, pitted; *G* and *H*, annular in an unstretched element (*G*), and in a stretched element (*H*). *A–F*, from an *Avena* seedling; *G* and *H*, from a *Zea* seedling. (*A–F*, ×450; *G*, *H*, ×420. *A–F*, drawn from photomicrographs in Goodwin, 1942.)

Figure 5.15

Fibres: Fibres may be from the xylem, or from other tissues (extraxylary fibres). These latter may arise from a number of tissues, phloem, cortex and pericycle. The exact phylogenetic status of these fibres is usually of little interest to the analyst. Perivascular fibres describe those that occur in a ring around the vascular system, and if this ring arises from the pericycle, may be called pericyclic. Cortical and phloem fibres arise from adjacent areas and do not need to be distinguished for our purposes. One may also see extraxylary fibres described as bast or bast fibres (see Figure 5.16).

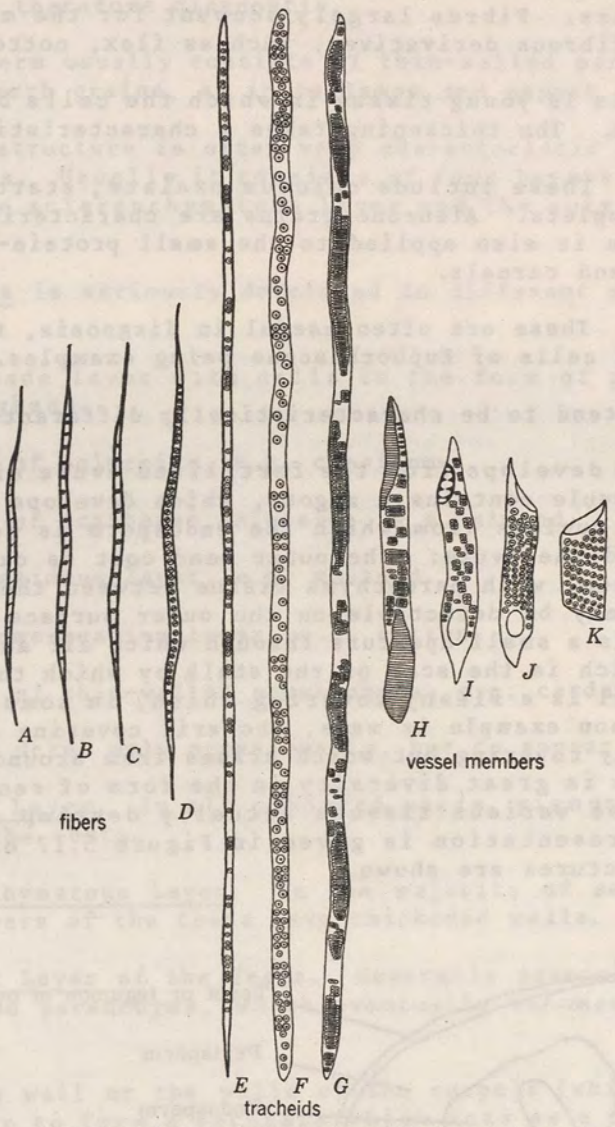


Illustration of the main lines of specialization of the tracheary elements and fibers. *E–G*, long tracheids from primitive woods. *E* and *F* have circular bordered pits; *G* has elongated bordered pits in scalariform arrangement.

Figure 5.16

Pits: These are areas on tracheids, vessels and sieve-tubes where no thickening has taken place. (See Figure 5.16).

Tracheids: This is different from a vessel in that the end-walls of the cell are not perforated. Tracheids occur in xylem tissue. The conduction of water, although generally in vessels, may occur through tracheids, which consist of single cells, prosenchymatous and usually with bordered pits or spiral thickening. Tracheids are found in leaves and in wood. Coniferous wood consists of long tracheids which look rather like fibres with bordered pits. Thus it is a fairly simple matter to classify sawdust as from soft or hard wood.

Sclerenchyma: In this type of cell, lignin has been deposited in and around the cell-wall. The tissue is identified by being stained scarlet with phloroglucinol and hydrochloric acid. Sclereids (stone-cells) and fibres are common forms. The presence of stone cells accounts for the "gritty" feeling of the center of pears. Fibres largely account for the mechanical strength of plants and their fibrous derivatives, such as flax, cotton, sisal.

Collenchyma: This is young tissue in which the cells become thickened, even though still alive. The thickening takes a characteristic form.

Cell Inclusions: These include calcium oxalate, starch, aleurone (protein) grains and oil droplets. Aleurone grains are characteristic of oil-containing seeds and the term is also applied to the small protein-containing granules of leguminous seeds and cereals.

Secretory Cells: These are often useful in diagnosis, the laticiferous (latex or milk producing) cells of Euphorbiaceae being examples.

Starches: These tend to be characteristically different in different plants.

The Seed: A seed develops from the fertilized ovule situated at the base of the flower. The ovule contains a zygote, which develops into the embryo, and a primary endosperm nucleus from which the endosperm is formed. Several layers (or coats) surround the ovule: the outer seed coat is called the testa and the inner is the tegumen, with parenchyma tissue between them called the nucellus. Structures which may be detectable on the outer surface of the seed are: The micropyle, which is a small aperture through which air and water enter the seed and the hilum, which is the scar of the stalk by which the seed was attached to the fruit. An aril is a fleshy covering which, in some seeds, develops from the hilum. A common example is mace, the aril covering a nutmeg. An arillode is a similar fleshy covering but which arises from around the micropyle, as in cardamom. There is great diversity in the form of seeds, depending in part upon whether these various tissues actually develop and at what rate. A diagrammatic representation is given in Figure 5.17 of a seed in which all these tissues/structures are shown.

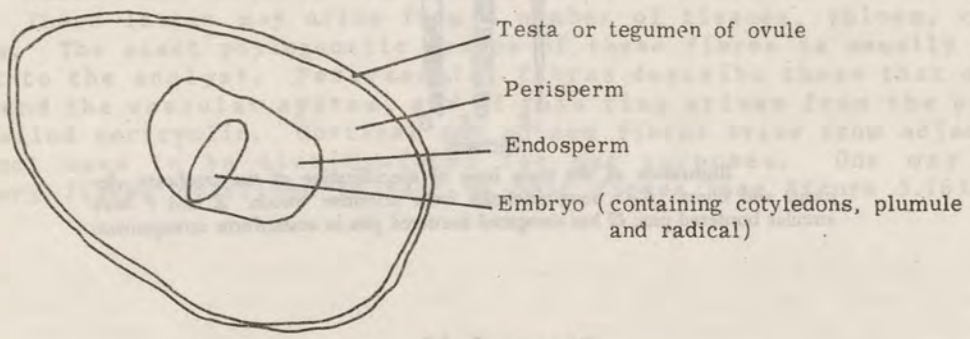


Figure 5.17

The various seed parts (as represented in Figure 5.17) are:

Embryo: The embryo possesses the rudiments of the plant into which it will develop, that is, the cotyledon or cotyledons, which sometimes contain storage material and often have the cellular structure of the leaves into which they will develop; a hypocotyl which develops into a root and an embryonic shoot, the plumule, which develops into the stem.

Endosperm: Generally composed of cellulose-walled parenchyma cells which contain food, such as the protein reserves, aleurone grains. They are only found in seeds and are therefore diagnostic.

Perisperm: The perisperm usually consists of thin-walled parenchymatous cells containing abundant starch grains, as in cardamom and pepper.

Testa: The seed coat structure is often very characteristic and may be useful in identifying powders. Usually it consists of four layers: the epidermis, the pigment layer, the sclerenchymatous layer and the nutrient layer of the testa.

1. The Epidermis is variously developed in different seeds and may take the following forms:

   a. A palisade layer with cells in the form of polygonal prisms, e.g. soybean.

   b. A layer of sclereids, e.g. capsicum.

   c. A layer of scattered sclereids, e.g. almond.

   d. A mucilaginous layer, e.g. mustard.

   e. A trichome-bearing layer, e.g. cotton.

   f. A layer of thin-walled prosenchyma, e.g. cardamom.

   g. Stomata occur only occasionally, but do appear in cotton.

2. The Pigment Layer. In all coloured seeds, pigment is deposited in one of the layers of the testa.

3. The Sclerenchymatous Layer. In the majority of seeds, the cells of one or more of the layers of the testa have thickened walls.

4. The Nutrient Layer of the Testa. Generally several cells thick, and consists of thin-walled parenchyma, which eventually becomes flattened and is full of starch.

The Fruit: The ovary wall or the walls of the carpels (which enclosed ovules in the flower) develop to form a pericarp which acts as a case for the seeds and is called a fruit. The fruit can either be dry as in caraway seeds, or fleshy as in apple or cherry. The pericarp differentiates into endocarp, mesocarp and exocarp (see Figure 5.18).

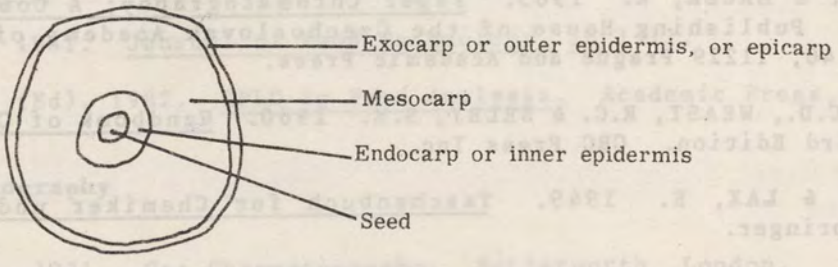


Figure 5.18

Fruits may be simple (from one carpel, e.g. prune, olive), or aggregate (raspberry), or compound (from a number of flowers, e.g. fig, mulberry, red pepper).

The epicarp and endocarp may consist of only an epidermal layer and the epicarp may have a few stomata. The epicarp of pepper and cubeb consist of the epidermis plus hypodermal layer of sclereids and parenchyma. It is the endocarp of umbelliferous fruits that forms the parquetry layer which is important in their identification. In citrus fruits the flavedo or rind forms the epicarp, the white tissue or albedo forms the mesocarp and the edible flesh is composed of modified hairs derived from the endocarp. The mesocarp may be succulent as in tamarinds or pithy as in colocynth or it may be composed of a spongy parenchyma as in lobelia.

Preparing Specimens and Clearing Agents

Starchy specimens such as flours are usually mounted in alcohol or methanol:glycerol (1:1). If it is necessary to prepare sections of starchy reference specimens such as certain seeds, it is convenient to soak them overnight in glycerol to soften the specimen. Starch granules gradually swell in water. Water is therefore not a suitable medium in which to examine them. Non-starchy powders may be mounted in a drop of water for a preliminary examination. It is important to take only a tiny amount of the sample so that individual particles are completely separated from each other. If the sample is too heterogeneous to allow all of the different elements present to appear on one slide, it is better to prepare several slides rather than try to crowd a confusing mass of the sample on a single slide.

Cellulosic elements in plants are resistant to solvents such as chloral hydrate and dilute sodium hydroxide solution and it is therefore convenient to boil a small amount of the sample with one of these clearing agents. Chloral hydrate solution is usually prepared by dissolving 7 g of the solid in 5 ml of water. 2% sodium hydroxide is also effective. If the amount of cellulosic material present in a sample is small it may be necessary to centrifuge the cleared specimen and examine the deposit. Schulze's maceration fluid (potassium chlorate dissolved in concentrated nitric acid) is effective in breaking woody tissue into its component cells. The specimen is usually left to soak in the liquid at room temperature. Sodium hypochlorite solution (or a solution of bleaching powder) is useful in lightening the colour of material such as tea. Some solutions are used diagnostically. Iodine solution (in aqueous KI) gives a blue colour with starch cells and a brown colour with aleurone grains. Fat-soluble dyes such as Sudan III and Sudan Blue are absorbed by any oil globules present in the specimen (examined in alcohol). Hydrochloric acid and phloroglucinol (1% in alcohol) stain lignin red. Corallin soda stains mucilage pink.

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General History	JAPA 8(3), 74-80 (1970)
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Fats & Oils	JAPA 14(3), 113-117 (1976)
Milk & Milk Powder	JAPA 15(1), 33-37 (1977)
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Water	JAPA 17(2), 79-81 (1979)
Bakery & Allied Products	JAPA 17(3), 111-114 (1979)
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Dairy Products	JAPA 19(3), 105-111 (1981)
Butter & Margarine	JAPA 20(2), 51-54 (1982)
Fruits & Nuts	JAPA 20(1), 25-30 (1982)
General Kitchen Products	JAPA 20(3), 99-101 (1982)
Meat & Fish	JAPA 21(1), 29-33 (1983)
Vegetable Products	JAPA 21(3), 93-98 (1983)

6. FOOD ADDITIVE METHODS

6.1 PRESERVATIVES

Preservatives can be generally defined as chemicals inhibiting or reducing the growth of bacteria or other microbial flora. Substances which have an antioxidant function may also serve as preservatives, but are dealt with in another section.

National legislation relating to preservatives is extremely varied but in general, it is usually necessary to carry out qualitative tests in order to ensure that non-permitted preservatives are absent and quantitative analysis to ensure that permitted preservatives do not exceed any legal limit there may be.

In addition to the chemical preservatives discussed here, it should not be forgotten that salts, especially common salt itself, and acids such as vinegar, sodium diacetate and fruit acids all have a preservative action. Non-specific analysis such as ash and acidity determinations are usually sufficient to establish that their use has not been excessive. Exception can reasonably be taken to the presence of an excessive amount of alum.

One of the most common preservatives used for canned or bottled foods is benzoic acid, usually as its sodium, potassium or calcium salt. Benzoic acid retards the growth of moulds and yeasts and works best in food products having an acid pH of 2.5 to 4.0. Its effectiveness decreases dramatically when the pH rises above 5.0.

A group of effective benzoic acid derivatives are compounds commonly referred to as "parabens". These are methyl, ethyl or propyl p-hydroxy-benzoates. Their main usefulness is that they are effective over a wider range of pH than benzoic acid and are quite stable.

Sorbic acid (2,4-hexadienoic acid) is often used in dairy products such as cheese to inhibit mould growth, as it does not affect the lactobacilli present. Like benzoic, it is added as the sodium, potassium or calcium salt.

Another widely used preservative is sulphur dioxide. It is added to foods primarily as the sulphite, bisulphite or metabisulphite of sodium, potassium or calcium. It is primarily a preservative, but has antioxidant properties as well and is often used to prevent browning.

Analytical methods for other chemicals having preservative action, which are used only with specific foods, are given in the Manual, "Food Analysis: General Quality, Adulteration, Identity". These include nitrite/nitrate in meat products and boric acid in fish.

BENZOIC ACID
(Quantitative Method)

PRINCIPLE

Benzoic acid may be extracted from a liquid sample using chloroform, after saturating with salt, or may be steam distilled. Permanganate is used to destroy unwanted acidic substances. Spectrophotometry is the preferred method to quantify, but the extracted benzoic acid can be titrated using standard sodium hydroxide solution (1 ml of 0.05 N NaOH = 0.0072 gm sodium benzoate).

APPARATUS

1. Separatory funnels.
2. Steam distillation apparatus.
3. Spectrophotometer, preferably double beam.

REAGENTS

1. Ortho-phosphoric acid.
2. Sodium chloride.
3. 1 N sodium hydroxide.
4. 0.1 N sodium hydroxide.
5. 5% potassium permanganate.
6. Sodium sulphite.
7. Diethyl ether/petroleum ether BP 40-60° (1:1).
8. 10% sodium hydroxide.
9. Dilute hydrochloric acid (1+3).
10. Chloroform.
11. Diethyl ether.
12. Benzoic acid standard in ether (5 mg/100 ml).

PROCEDURE

Solvent Extraction

Mix sample thoroughly, grinding if solid or semi-solid. Transfer 150 ml or 150 g to a 500 ml volumetric flask, add enough sodium chloride to saturate the water in the sample, make alkaline to litmus paper with 10 % sodium hydroxide solution and dilute to volume with saturated sodium chloride solution. Shake thoroughly and let stand for at least two hours, shaking frequently and filter. If the sample contains large amounts of fat, portions of which may contaminate filtrate, add a few ml of 10% sodium hydroxide to filtrate and extract with ether before proceeding with the determination.

For products containing alcohol, make 250 ml of sample alkaline to litmus paper with 10% sodium hydroxide and evaporate on a steam bath to about 100 ml. Transfer to a 250 ml volumetric flask, add 30g

sodium chloride and shake until dissolved. Dilute to 250 ml with saturated sodium chloride solution, let stand for at least 2 hours, shaking frequently and filter.

For salted or dried products such as fish, wash 50 g of ground sample into a 500 ml volumetric flask with water. Make slightly alkaline to litmus paper with 10% sodium hydroxide solution and dilute to volume with water. Let stand for at least 2 hours, shaking frequently and filter. Pipette as large a measured volume of filtrate as possible (300 ml) into a second 500 ml volumetric flask and add 30 g of sodium chloride for each 100 ml of solution. Shake until salt dissolves and dilute to volume with saturated sodium chloride solution. Mix thoroughly and filter off precipitated protein and other extraneous matter.

Pipette 100-200 ml of filtrate into a separating funnel. Neutralise to litmus with dilute hydrochloric acid and add 5 ml excess. With salted fish, protein usually precipitates with the acid but this does not interfere with the extraction. Extract carefully with chloroform, using successive portions of 70, 50, 40 and 30 ml. To avoid formation of emulsion, shake cautiously each time using a rotary motion. The chloroform layer usually separates readily after standing for a few minutes. If an emulsion forms, break it by stirring the chloroform layer with a glass rod, by drawing off into a second separator and giving one or two sharp shakes from one end of separator to the other, or by centrifuging for a few minutes. As this is a progressive extraction, carefully drain as much clear chloroform as possible after each extraction, but do not drain any of the emulsion. If this precaution is taken, the chloroform layer need not be washed.

Transfer the combined chloroform extracts to a porcelain evaporating dish, rinse container several times with a few ml of chloroform, and evaporate to dryness at room temperature in a current of dry air.

Steam Distillation

Place 30-100 g sample in a 500 ml steam distillation flask. Add sufficient water and an excess of salt (40 g/100 ml). Make distinctly acid with orthophosphoric acid and rapidly steam distil 500 ml into a flask containing 10 ml of 1 N sodium hydroxide. Wash down the condenser with 25 ml of 0.1 N sodium hydroxide into the receiving flask. Evaporate the alkaline distillate down to about 20 ml on a steam bath. Allow to cool to about 45°C and add potassium permanganate solution until a pink colour persists after stirring. Decolourise with sodium sulphite and add sufficient dilute sulphuric acid to dissolve the precipitated manganese dioxide and make the liquid acid.

Transfer to a stoppered separatory funnel, saturate with salt (33 g/100 ml water) and extract the benzoic acid with four successive 15 ml portions of ether/light petroleum. Combine and transfer all extracts to a beaker and evaporate off the solvent by means of a current of dry air, while holding the beaker in a water bath at 30°C.

Determination

Dissolve the residue from either the solvent extraction or steam distillation in sufficient diethyl ether to make a solution of 5 g equivalent of sample per 100 ml. (This assumes a sample concentration of 0.1% benzoic acid. Adjust volumes if the expected amount differs from this.)

Scan both the sample and benzoic standard solutions between 240 and 300 nm, using ether as the reference in each case.

Record the absorbances at the maxima of 272 nm and the two minima of 267.5 and 276.5 nm. Also compare the overall scans of both standard and sample. They should have identical shapes, even though they may differ in magnitude.

CALCULATION

$$\text{ppm benzoic acid} = \frac{As}{Ax} \times \frac{(.05)(1000)(V)}{(w)}$$

where:

As = absorbance of the sample solution at 272 nm minus the average of the absorbances at 267.5 and 276.5.

Ax = absorbance of the standard solution calculated as for the sample.

V = ml of ether used to dissolve sample residue.

w = g equivalent of sample in final solution.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 24.024-.028.

BENZOIC ACID
(Qualitative Identification)

PRINCIPLE

The UV scan of benzoic acid, used in the quantitative procedure, is distinctive but is not an absolute identification. In the event that confirmation of identification is needed, use the following thin-layer chromatography method.

APPARATUS

1. Thin-layer apparatus including tanks, plates and spotting pipettes.

2. Viewing cabinet with shortwave (254 nm) UV light.

REAGENTS

1. Kieselguhr G.

2. Silica gel GF 254.

3. n-Hexane:acetic acid solution (96 + 4).

4. Benzoic acid standard solution (1 mg/ml in ether).

PROCEDURE

Prepare thin-layer plates as follows: In 250 ml conical flask, mix 10 g each of kieselguhr G and silica gel GF 254. Add 45 ml of water and shake 30 seconds. Set applicator at 0.25 mm. Apply mixture to 5 glass plates. Air dry for 10 minutes and dry in an oven at 100°C for 1 hour.

Dissolve the residue extracted from the sample (see "Quantitative Method") in ether (not more than 2 ml). Spot 50 µl sample solution, using 10 µl syringe (5x) on prepared plate. Also, spot 50 µl benzoic acid standard solution. Spot sample(s) and standard about 2.5 cm from bottom edge of plate and 4 cm apart, beginning 2.5 cm in from side edge. Draw a line across the plate 10 cm above the base line.

Place plate in chromatographic tank containing 250-300 ml mobile solvent (n-hexane:acetic (96 + 4)). Develop chromatogram for 10 cm. Remove from tank and air dry for 5 minutes.

Observe the plate under UV shortwave light (254 nm). Sample and standard benzoic acid appear as dark blue-purple spots on light fluorescent background. With a sharp point (e.g. pin or pencil) circle spots from edge of each spot. (This gives exact location of each acid when plate is removed from the light). Calculate and compare RF value of standard and sample solution.

INTERPRETATION

If the sample streaks or otherwise does not present a discrete spot, the sample residue may have to be further purified by dissolving in an alkaline aqueous solution, extracting with chloroform (discarding the chloroform), then making distinctly acid (at least pH 4) and re-extracting with chloroform. Evaporate the chloroform, dry the residue overnight in a desiccator and repeat the thin-layer identification analysis.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 20.029-.033.

PARABENS
(Semi-quantitative Method)

PRINCIPLE

"Parabens" are p-hydroxybenzoates. This method determines their presence using thin-layer chromatography. Their concentration can be semi-quantitatively estimated by comparing the sample to standards of differing concentration.

APPARATUS

1. Separatory funnels.

2. Thin-layer chromatography apparatus.

3. Viewing cabinet with shortwave (254 nm) UV light.

REAGENTS

1. Mobile solvent-toluene:methanol:acetic acid (90:16:8).

2. 2% standard solutions in ether of methyl, ethyl and propyl hydroxy-benzoates and the free acid.

3. Deniges reagent: mix 5 g yellow mercuric oxide with 40 ml water, cool in ice/salt and very slowly add freezing-cold concentrated sulphuric acid (20 ml) with stirring. Add another 40 ml of water.

4. TLC plate coated with silica gel containing a pigment fluorescent under short wave UV.

5. Sulphuric acid 10% by volume.

6. 2% sodium nitrite solution, freshly prepared.

7. Sodium sulphate.

8. Methanol.

PROCEDURE

Add 5 ml 10% sulphuric acid to 10 g of food and grind with sodium sulphate in a mortar until the sample is dry. Add about as much sodium sulphate again. Grind the powder with small successive quantitites of ether and decant the ether through a filter paper into a flask. Evaporate the ether at as low a temperature as possible and dissolve the residue in 0.5 ml of methanol. Spot 20 microlitres of sample and about the same amount of the 2% standards on the TLC plate and run the chromatogram in the toluene:methanol:acetic acid solvent.

p-Hydroxybenzoates show black under short-wave UV. Interfering substances may be present so caution should be used in interpreting the results. Mark any quenched areas lightly with a pin, and spray lightly with Deniges reagent. p-Hydroxybenzoates give a white spot, visible by its different reflectivity from the background. Heat at 100°C for five minutes and spray lightly with fresh 2% sodium nitrite solution. Any spots become red.

INTERPRETATION

If the object of the test is to confirm that the amounts of any p-hydroxybenzoates present are below a legal maximum, the quantities of standards spotted can be chosen to correspond to that maximum. Sample spots of lower intensity are taken to indicate compliance. If quantitative analysis is

necessary the method of Thackray and Hewlett (1) may be followed. Recoveries are about 85% except with certain samples such as coffee essence (about 50%). Dickes discusses false positives obtained by this method. The solvent extraction is necessary as, unlike benzoic and sorbic acid, the free acid is not steam-volatile and the esters are only partially so.

REFERENCE

Dickes, G.T., 1965. Journal of the Association of Public Analysts 3, 73-75.

SORBIC ACID

PRINCIPLE

This analysis involves steam distillation of the free acid, oxidation to malonaldehyde using dichromate and reaction with thiobarbituric acid to form a red complex. This is determined spectrophotometrically at 532 nm.

APPARATUS

1. Steam distillation apparatus.

2. Volumetric flasks, 50 ml, 100 ml, 500 ml, 1 litre.

3. Boiling water bath.

4. Spectrophotometer.

REAGENTS

1. Magnesium sulphate, heptahydrate.

2. Sulphuric acid solutions, 1 N and 0.01 N.

3. Sodium hydroxide solution, 1 N.

4. Dichromate solution: mix equal volumes of 0.3 N H_2SO_4 and a solution of 0.5 g $K_2Cr_2O_7$ in one litre of water. Prepare fresh as needed.

5. Thiobarbituric acid (TBA) solution: dissolve 0.5 g TBA in 25 ml water + 20 ml 0.5 N NaOH. Add 11 ml 1 N HCl and dilute to 100 ml with water.

PROCEDURE

Weigh 50 g sample into a 1 litre steam distillation flask. Add 100 g $MgSO_4 \cdot 7H_2O$ and 100 ml 1 N H_2SO_4. Place 10 ml 1 N NaOH in the steam distillation apparatus receiver. Steam distill rapidly. (Note - do not heat the distilling flask). Collect about 450 ml distillate in about 30 min.

Cool and transfer the distillate to a 500 ml volumetric flask. Add 15 ml 1 N H_2SO_4 and make to volume with water. Mix. Pipette 2 ml into a test tube and add 2 ml of the dichromate solution. Heat in a boiling water bath for 5 min. Then cool. Add 2 ml TBA solution and heat in a boiling water bath 10 min. Cool rapidly and transfer to a 50 ml volumetric flask with water. Make to volume with water. Measure the absorbance of the solution at 532 nm using a 1 cm cuvette, and water as the reference.

Prepare a standard curve as follows: Dissolve 1.0 g of sorbic acid in a small volume of 1 N NaOH and dilute to 1 litre with water. This is the Stock Solution (1 mg/ml). Prepare a blank and four working standard solutions by first pipetting 25.0 ml of the stock solution to a 500 ml volumetric flask (50 µg/ml) and diluting to volume with water. Next, pipette 0.0, 10.0, 20.0, 50.0 and 80.0 of this solution into five 100 ml volumetric flasks and dilute to volume with water (range 0, 5, 10, 25 and 40 µg/ml). Pipette 2 ml of each of the working standards and blank into five test tubes and continue as in the above procedure, starting at addition of dichromate. Plot absorbance vs µg sorbic acid for a standard curve. (µg sorbic = 0, 10, 20, 50, 80 in the 2 ml aliquots).

CALCULATION

$$\text{Sorbic acid ppm} = \frac{A}{S} \times \frac{500}{2}$$

where:

A = µg sorbic acid corresponding to the sample absorbance, taken from the standard curve.

S = sample weight in g.

REFERENCE

Carr, W. and Smith, G.A., 1964. Journal of the American Pharmaceutical Association 2 (2), 37-43.

SULPHUR DIOXIDE

PRINCIPLE

Sulphur dioxide, in solution as sulphurous acid, inhibits the growth of mold, yeasts and aerobic bacteria. It also prevents the browning of fruits and vegetables due to enzymic reactions. It assists in conserving Vitamin C, but destroys Vitamin B1, by cleavage of the molecule. The analysis of sulphur dioxide in foods involves distillation using nitrogen as a carrier, collection in an oxidizing solution (hydrogen peroxide), thus converting sulphurous acid to sulfuric, and a titration of the acid. Two traps are used for distillate collection to ensure all sulphur dioxide is removed from the nitrogen carrier stream. This method uses phosphoric instead of hydrochloric for initial acidification in order to slow the release of sulphur dioxide.

APPARATUS

1. Distillation apparatus (see Figure).

2. Nitrogen gas (from a cylinder, with regulator).

3. Burette.

REAGENTS

1. Methanol.

2. Hydrogen peroxide solution, 3.0% (w/v): dilute 10 ml of 30% H_2O_2 to 100 ml with water. Prepare daily.

3. Mixed indicator solution: mix equal volumes of 0.03% methyl red (in ethanol) and 0.05% methylene blue (also in ethanol) and filter.

4. Sodium hydroxide solution, 0.01 N.

5. Phosphoric acid, concentrated (88%).

PROCEDURE

Introduce sample and distilled water into the distillation flask. The amount of each is dependent on the expected sulphur dioxide content, as noted below:

Expected SO_2 (ppm)	Sample weight (g)	Water volume (ml)
10	50	20
10 - 100	25	30
100	10	40

Add 50 ml methanol and mix by swirling. Introduce 20 ml of the H_2O_2, 50 ml distilled water and a few drops of the mixed indicator into the conical receiving flask. Add a few drops of 0.01N NaOH to produce a green color. Introduce 10 ml of neutralized H_2O_2 solution to the second receiver. (Make sure the receiving tubes are below the level of the liquid in each trap). Connect the nitrogen and adjust the flow to about one bubble per second. Add 15 ml H_3PO_4 to the distillation flask and quickly attach the condenser. Heat rapidly to a boil, then reduce heat and simmer for 30 minutes. Detach receivers (rinsing the tubes with water in the process) and combine their contents. Titrate the H_2SO_4 using 0.01N NaOH to a green endpoint.

CALCULATION

$$\text{Sulphur dioxide (ppm)} = \frac{(V)\ (N)\ (32)\ (1000)}{S}$$

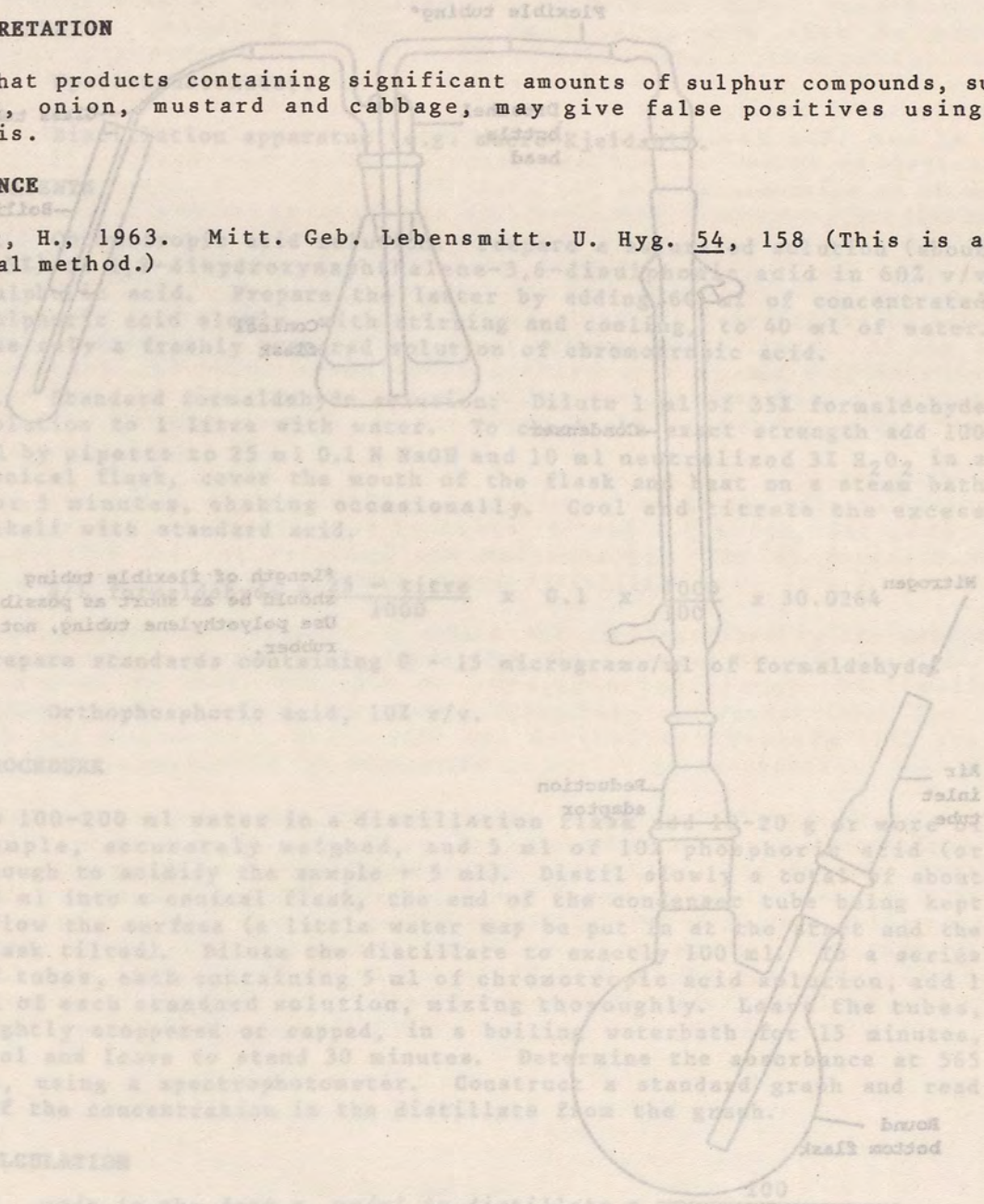
Where: V = ml of NaOH used to titrate.
N = normality of NaOH.
S = g of sample.

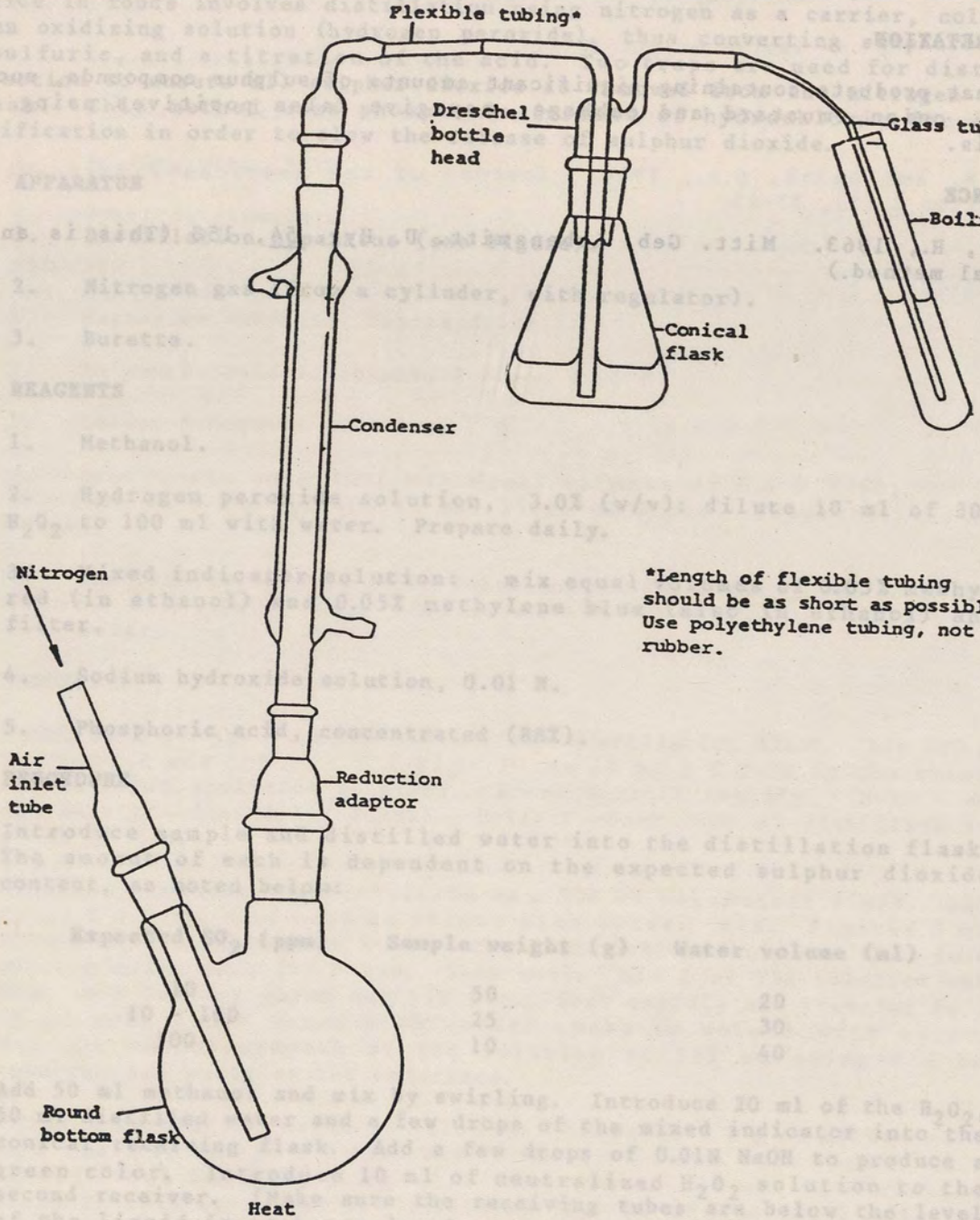
INTERPRETATION

Note that products containing significant amounts of sulphur compounds, such as garlic, onion, mustard and cabbage, may give false positives using this analysis.

REFERENCE

Tanner, H., 1963. Mitt. Geb. Lebensmitt. U. Hyg. 54, 158 (This is an EEC official method.)





Sulphur Dioxide Distillation Apparatus

FORMALDEHYDE

PRINCIPLE

The use of formaldehyde in food is prohibited or severely restricted in most countries, but it may be illegally used from time to time. The method involves distillation of the formaldehyde, reaction with chromotropic acid and spectrophotometric determination.

APPARATUS

1. Spectrophotometer.

2. Distillation apparatus (e.g. macro-Kjeldahl).

REAGENTS

1. Chromotropic acid solution: Prepare a saturated solution (about 0.5%) of 1,8-dihydroxynaphthalene-3,6-disulphonic acid in 60% v/v sulphuric acid. Prepare the latter by adding 60 ml of concentrated sulphuric acid slowly, with stirring and cooling, to 40 ml of water. Use only a freshly prepared solution of chromotropic acid.

2. Standard formaldehyde solution: Dilute 1 ml of 35% formaldehyde solution to 1 litre with water. To check the exact strength add 100 ml by pipette to 25 ml 0.1 N NaOH and 10 ml neutralized 3% H_2O_2 in a conical flask, cover the mouth of the flask and heat on a steam bath for 5 minutes, shaking occasionally. Cool and titrate the excess alkali with standard acid.

$$\text{g/L formaldehyde} = \frac{25 - \text{titre}}{1000} \times 0.1 \times \frac{1000}{100} \times 30.0264$$

Prepare standards containing 0 - 15 micrograms/ml of formaldehyde.

3. Orthophosphoric acid, 10% v/v.

PROCEDURE

To 100-200 ml water in a distillation flask add 10-20 g or more of sample, accurately weighed, and 5 ml of 10% phosphoric acid (or enough to acidify the sample + 5 ml). Distil slowly a total of about 90 ml into a conical flask, the end of the condenser tube being kept below the surface (a little water may be put in at the start and the flask tilted). Dilute the distillate to exactly 100 ml. To a series of tubes, each containing 5 ml of chromotropic acid solution, add 1 ml of each standard solution, mixing thoroughly. Leave the tubes, lightly stoppered or capped, in a boiling waterbath for 15 minutes, cool and leave to stand 30 minutes. Determine the absorbance at 565 nm, using a spectrophotometer. Construct a standard graph and read off the concentration in the distillate from the graph.

CALCULATION

$$\mu\text{g/g in the food} = \mu\text{g/ml in distillate} \times \frac{100}{\text{weight of sample}}$$

This test may be used as only qualitative, omitting the preparation of standards. Other qualitative tests will be found in standard texts. Results are likely to be low due to incomplete recovery as a result of polymerization and decomposition after incorporation of the formaldehyde into the food.

REFERENCE

Kleinert, T. and Srepel, E., 1948. Mikrochem. 33, 328-332.

6.2 ANTIOXIDANTS

Antioxidants by definition are designed to retard deterioration of a food through the action of atmospheric oxygen. They may also incidentally have a preservative action, but are used primarily for their antioxidant function.

Antioxidants commonly found in fats, oils and the fatty fractions of foods include the gallates (mainly propyl, octyl and dodecyl), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT). Tocopherols and their esters, usually acetate or palmitate, may also be present. Nordihydroguaiaretic acid, ascorbyl palmitate, ethoxyquin, 2,4,5-trihydroxybutyrophenone, guaiacol from guaiac resin, tertiary-butyl hydroquinone (TBHQ), thiodipropionic acid and its dilauryl and distearyl esters and 4-hydroxymethyl-2,6-di-tert-butylphenol (4-hydroxy BHT) may be found occasionally either as a result of incorporation in food or migration from packaging material. Most of them are permitted in some countries up to certain limits. Many amines and aminophenols such as diphenyl paraphenylenediamine are very effective antioxidants, used in other products such as gasoline and rubber. However, they should not be found in food.

If a permitted antioxidant is found by a qualitative test it may be necessary to establish that it is present at a level below a legal limit. If the amount is well below the limit a semi-quantitative test by TLC may be enough to establish that the sample gives a spot of less colour intensity than that of a standard amount corresponding to the legal limit. Otherwise a quantitative method must be used.

Quantitative tests for antioxidants are described in the Official Methods of Analysis of the AOAC (1984) and by Amato (2) and Takeshita, Sakogami & Itoh (3). Cantafor (4) has reviewed the methodology. TLC of antioxidants is described by Schneider (5) and EEC method 992/VI/71.

Quantitative methods for gallates, BHA & BHT are described in the publication of the Association of Public Analysts (U.K.)(6). Page & Kennedy (7) describe a GLC-EC method for BHA, TBHQ and PG. The colorimetric microestimation of antioxidants is detailed by Jayaraman, Vasundhara and Panhar (8). The papers by Lamb and Woller (9), Holdt (10) and Groebel and Wessels (11) are also useful. GLC determination of ethoxyquin in apples is described by Wirell (12).

For qualitative methods for BHA see Anglin, Mahan and Chapman (13), Amato (2) and Dilli and Roberts (14) who describe a spectrofluorimetric technique. For BHT see Sahasrabundhe (15), Selmeci and Aczel (16), and for the two taken together see Keen and Green (17), Sato and Kawamira (18), Takahashi (19) and Hartman and Rose (20), the two latter describing GLC methods. Gallates may be determined by the method of Cassidy and Fisher (21), or the GLC method of Wachs and Gassmann (22). Berk and Bielecki (23) describe the determination of antioxidants in essential oils. The IUPAC-AOAC liquid chromatography method was first described by Page (24)(25).

GALLATES

PRINCIPLE

The gallates are extracted with 95% methanol. An aliquot of the methanolic extract is treated with acetone and finely powdered ammonium sulphate and the blue colour compared with standards.

APPARATUS

1. Spectrophotometer or colorimeter.

REAGENTS

1. 95% methanol. Mix 95 ml methanol with 5 ml water.
2. Calcium carbonate, finely divided.
3. Acetone.
4. Ammonium sulphate, finely powdered.
5. Standard n-propyl, n-octyl or n-dodecyl gallate. Dissolve 0.100 g in 95% methanol and dilute to 100 ml (1 mg/ml). Prepare dilute standards in the range 5-50 mg/L.

PROCEDURE

Vigorously shake 10 g of warm liquid sample or melted fat with 25 ml of 95% methanol for one minute in a centrifuge tube. Place in a water-bath at 40-50°C and allow to separate for about 15 minutes (separation into clear layers is unlikely and unnecessary). Pour the upper layer into a 50 ml volumetric flask. Repeat the extraction with 20 ml of 95% methanol, again transfer the upper layer to the flask and dilute to the mark. Add 1 g of calcium carbonate, shake and filter through a paper (Whatman No. 1 or equivalent), rejecting the first few ml. of filtrate. The amount of calcium carbonate is not critical but must be enough to ensure a clear filtrate.

Pipette 10 ml of filtrate and add 1 ml of acetone and about 10 mg of finely divided ammonium sulphate. Shake for one minute. After half an hour measure the absorbance of the blue colour at 580 nm in a 1 cm cell and compare with the colour developed from 10 ml of dilute standard in 95% methanol treated similarly.

CALCULATION

Absorbance values for the three commonest gallate antioxidants are as follows:

	Approximate absorbance using 10 ppm solution in above test	absorptivity	molecular weight	molar absorptivity (absorbance x M. Wt)
Propyl gallate	0.1777	17.68	212	3749
Octyl gallate	0.140	14.01	282	3952
Dodecyl gallate	0.116	11.55	338	3905

The close similarity of the molar absorptivities means that results may, without serious error, be expressed in mg of the gallate used as standard per kilogram of sample even if another gallate or a mixture is present.

$$\text{Gallates in mg/kg of sample} = \frac{\text{sample absorbance}}{\text{standard absorbance}} \times \text{standard in mg/L} \times 50/10$$

Enough standards should be prepared to bracket the sample concentration.

REFERENCE

Cassidy, W. and Fisher, A.J., 1960. Analyst 85, 295-7.

BHA

PRINCIPLE

BHA (butylated hydroxyanisole) is extracted from a sample with 95% methanol and reacted with Gibb's reagent to form a stable indophenol colour.

APPARATUS

1. Spectrophotometer.

REAGENTS

1. 95% v/v Methanol.

2. 0.5% Sodium tetraborate decahydrate.

3. 2,6-Dichloro-p-benzoquinone-4-chloroimine (Gibb's reagent) 0.01% in 95% methanol.

4. n-Butanol.

5. BHA standard, 25 mg/L in 95% methanol.

PROCEDURE

Shake 10 g of warm liquid sample with 25 ml 95% methanol for one minute in a centrifuge tube. Place in a water bath at 40-50°C and let separate for 15 minutes (separation into clear layers is unlikely and unnecessary). Pour the upper layer into a 50 ml volumetric flask. Repeat the extractions with 20 ml of 95% methanol and again transfer the upper layer to the flask. Dilute the flask to the mark with 95% methanol.

Pipet 2 ml of this extract, add 2 ml of 95% methanol, 8 ml of borax solution and 2 ml of Gibb's reagent. After 15 minutes dilute to exactly 20 ml with n-butanol. Prepare a blank using 2 ml of 95% methanol and a standard using the 25 mg/L BHA solution. Read absorbances at 610 nm.

CALCULATION

$$\text{BHA in sample, in mg/L} = \frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}} \times 25 \times \frac{50}{10}$$

INTERPRETATION

The test may be made more sensitive by taking 4 ml of the methanol extract and omitting the 2 ml of 95% methanol. Other antioxidants, particularly gallates, reduce the intensity of the colour. For example, 200 mg/L of propyl gallate reduces the colour from 200 mg/L of BHA to about one half under the standard conditions of the test (concentrations calculated on the original sample). A table of corrections to be applied can be found in the referenced method.

REFERENCE

Association of Public Analysts (U.K.). 1962. The Detection and Determination of Antioxidants in Food.

BHT

PRINCIPLE

BHT (butylated hydroxytoluene) is steam-distilled, and determined by the colour reaction with o-dianisidine and sodium nitrite.

APPARATUS

1. Steam-distillation apparatus and steam generator.

2. Bath at 160°C. A one-litre beaker half-full of paraffin wax is convenient. An oil-bath may also be used.

3. Squibb-type separatory funnels of low actinic glass, or painted black. 60 ml.

4. 10 ml volumetric flasks of low actinic glass or painted black.

REAGENTS

1. Chloroform.

2. Magnesium chloride solution. Dissolve 100 g of the hexahydrate in 50 ml water.

3. o-Dianisidine solution. Dissolve 0.25 g in 50 ml methanol, add 100 mg activated charcoal, shake for 5 minutes and filter. Mix 40 ml of this clear solution with 60 ml of 1N HCl. Prepare daily and protect from light.

4. Sodium nitrite, 0.3% in water.

5. Standard solution of BHT, 500 mg/L. Dissolve 50 mg in methanol and dilute to 100 ml with methanol. Prepare working standards containing 1-5 mg/L by diluting with 50% v/v methanol.

PROCEDURE

Add 15 ml of the magnesium chloride solution to the distilling flask, add 5g of sample or less (preferably containing about 0.4 mg BHT). Preheat the bath for the distilling flask to 160°C $\pm$ 10. Adjust the steam generator to distill about 4 ml of water per minute. Maintain these conditions throughout the distillation. Connect the condenser and the steam generator to the distilling flask, and immediately immerse the latter in the bath. Steam distillation must be vigorous. Collect the first 100 ml of the distillate in a 200 ml volumetric flask containing 50 ml of methanol. Disconnect the distilling flask from the steam generator and remove the distilling flask from the bath. When the mouth of the condenser has cooled, disconnect it from the distilling flask and drain the water from the jacket. Wash the condenser with 5 ml portions of methanol adding the washings to the volumetric flask. Cool to room temperature and adjust the volume to 200 ml with methanol. Mix.

Thoroughly clean and dry three 60 ml Squibb-type separatory funnels of low actinic glass or painted black and mark them B, S, and X respectively. Into funnel B pipette 25 ml of 50% methanol (v/v); into funnel S pipette 25 ml of standard containing 1-3 µg of BHT per ml; and into funnel X pipette 25 ml of the 50% methanol solution (distillate) of the sample. To each funnel add 5 ml of dianisidine solution, stopper the funnel and carefully mix the contents. Then to each funnel add 2 ml of 0.3% sodium nitrite solution, stopper the funnels and thoroughly mix the contents. Let stand for 10 minutes,

then add 10 ml of chloroform to each funnel. Extract the coloured complex by vigorously shaking the funnels for 30 seconds. Let stand for 2-3 minutes to allow the two layers to separate completely.

Mark three 10 ml volumetric flasks of low actinic glass, B, S, and X respectively. Pipette 2 ml of absolute methanol into each flask. From the corresponding funnel draw off a sufficient volume of the chloroform (bottom layer) to reach the mark on the flask. Mix well. Read the absorbance of each solution in a suitable colorimeter or spectrophotometer set at 520 nm using a mixture of 2 ml methanol and 8 ml of chloroform as a blank. Avoid any unnecessary exposure of the solutions to light and air. 50 µg BHT should give an absorbance of about 0.39 in a 1 cm cell. Recovery should be 97 $\pm$ 2%.

CALCULATION

$$\text{BHT in sample, mg/kg} = \frac{A_s - A_b}{A_x - A_b} \times \mu\text{g/ml standard} \times \frac{200}{\text{g sample}}$$

INTERPRETATION

If extraction with 95% methanol is used as an alternative to steam distillation, only about half of the BHT is extracted so that doubling the result obtained by the o-dianisidine reaction gives an approximately quantitative answer. An extraction using acetonitrile is quantitative for BHT. Santoquin is the only reported interference.

REFERENCE

Association of Public Analysts (U.K.). 1962. The Detection and Determination of Antioxidants in Food.

ANTIOXIDANTS
(General Thin-Layer Chromatography Method)

PRINCIPLE

BHA, BHT and other antioxidants are extracted from oil or fat and separated by thin-layer chromatography. This method is semi-quantitative and the amounts of the various antioxidants can be estimated by comparing with standards.

APPARATUS

1. Separatory funnels, 250 ml.
2. Rotary vacuum evaporator.
3. Thin-layer plate spreader and developing tanks.
4. Spray bottle.

REAGENTS

1. Petroleum ether (40° - 60°BR).
2. Acetonitrile.
3. Methanol.
4. Silica Gel.
5. Citric acid, 1% in methanol.
6. Developing solution - (2+2+1) petroleum ether:benzene:glacial acetic acid.
7. Antioxidant standards - 0.1% in methanol.
8. Gibbs Reagent - 2,6-dichloro-p-benzoquinone-4-chloroimine, 0.5% in ethanol.

PROCEDURE

Weigh 10 g oil or fat and dissolve in 80 ml pet. ether. Transfer to a separatory funnel using about 20 ml pet. ether to wash. Add 25 ml acetonitrile and extract by inverting funnel several times. (Violent shaking may form an emulsion). Drain the acetonitrile into the rotary vacuum flask. Repeat the extraction with three more 25 ml portions of acetonitrile. Combine all extracts in the flask. Evaporate using vacuum at not over 40°C. Dissolve extract with 2 ml methanol.

Prepare 0.25 mm TLC plates using a slurry of 30 g silica gel G and 60 ml 1% citric acid solution. Air dry the prepared plates, then heat in an air oven for one hour at 130°C. Store the dried plates in a desiccator until required.

Line the developing tank with filter paper. Add solvent, close and allow to equilibrate 1-2 hours in the dark. Spot 10 µl and 20 µl portions of the sample extract on the prepared plate. Also spot 2, 5 and 7 µl portions of appropriate antioxidant standards of interest (corresponding to 2, 5 and 7 µg).

Develop the plate until the solvent front reaches 1 cm from the top. Remove from the tank, air dry and then spray with Gibb's Reagent. The following antioxidants will appear as coloured spots at about the R_F noted:

Antioxidant	R_F	Color
Propyl gallate	0.12	Brown
Octyl gallate	0.22	Brown
Dodecyl gallate	0.27	Brown
BHA	0.62	Brown-red
BHT	0.89	Brown-violet

Compare standard and sample spot size and intensity.

CALCULATION

If a semi-quantitative estimation is needed, compare the sample and standard spots (interpolate if the sample falls between two standards in intensity).

$$\text{ppm antioxidant} = \frac{1000 \times \mu g \text{ standard}}{5 \times \mu g \text{ sample}}$$

REFERENCE

Egan, H., Kirk, R.S. and Sawyer, R., 1981. Pearson's Chemical Analysis of Foods, 8th Ed., 88.

6.3 COLOURS

Colouring matters in food may be natural or synthetic, and may be acidic, neutral, or basic. Also, they may be conveniently classified as oil-soluble or water-soluble. They must be separated from the food before identification is attempted. Once separation is achieved by dyeing on to wool, quinoline extraction, use of a polyamide column, or other means identification is carried out by paper or thin-layer chromatography and confirmed by examination of the spectral absorption and by chemical tests. It should be possible to establish whether or not a colour belongs to those generally permitted but the identification of a non-permitted colour may well present considerable difficulty. Priority should be given to testing samples for a few common colours which are generally regarded as objectionable, mainly on grounds of carcinogenicity (suspected or confirmed), including auramine, the magentas (fuchsin, rosaniline), crystal violet, methyl yellow and the rhodamines. Some of these are basic dyes and therefore their presence will be shown by wool taking up the dye from ammoniacal solution. Some can occur as sulphonates and are therefore acidic water-soluble forms. Precautions for the use of carcinogenic substances should be taken with standards of suspected carcinogenic colours. Amaranth is broken down into naphthionic acid and 2-naphthol-3,6-disulphonic acid in the presence of salt and sugars. Erythrosine loses four atoms of iodine, forming fluorescein by the electrolytic action of tin and the latter dye may therefore sometimes be found in canned food. Some dyes occur as the calcium or aluminium "lakes", which can be decomposed to release the free dye by treatment with hydrochloric acid.

Great care must be taken in identifying a food colour which does not appear to be one in common use or on a permitted list. The presence of subsidiary dyes, impurities, co-extracted material from the food or too rigorous an extraction procedure may cause decomposition of the dye, and a resultant change of shade and R_f value during paper or thin-layer chromatography or interference in the spectral curve. For example, Red 2G will decompose to Red 10B in boiling dilute acid solution and Yellow RFS is converted to another dye by the wool-dyeing extraction procedure. Red 40 behaves similarly to Ponceau SX. It is therefore important in cases of doubt to mix dye and standard and check that they cannot be separated on paper or thin-layer by at least three solvents selected for their different polarities and acidities. Also examine UV-visible spectra, and carry out chemical tests to confirm identity. Spectra must be compared with authentic dye specimens, preferably more than one, derived from different sources.

A given colour has often been given different names by different manufacturers. There are even cases of two different dyes being given the same name by different manufacturers. A particular dye is identified unequivocally by its Colour Index number. Food colours presently or at one time permitted in the EEC have numbers starting at E100. Under U.S. legislation, colouring matters have been given numbers preceded by letters which indicate the permitted use. Although in most cases the dye.has subsequently been prohibited under U.S. law, it may still be identified in this way. For example, F.D. & C. Blue No. 1 indicates that the dye may be used in foods, drugs and cosmetics. Ext. D. & C. Red 9 was once permitted for external use only in drugs and cosmetics. The trivial names may include any numbers or letters in order to specify a particular dye. For example, Phloxine J and Phloxine ZB1 refer to Colour Index No. 45405 while Phloxine JN, Phloxine B and several more suffix letters refer to C. I. No. 45410.

WATER-SOLUBLE COLOURS
(Wool Dye Extraction)

PRINCIPLE

Acidic colour dyes are allowed to dye lengths of white defatted wool from dilute acetic acid solution. The colour is stripped from the wool with dilute ammonia solution and evaporated to small volume. The colouring matter is identified by an appropriate method. The dyeing and stripping may be repeated with fresh wool as a purification step. Basic colours are dyed onto wool in the presence of ammonia and stripped from the wool by dilute acetic acid.

REAGENTS

1. White defatted wool. Extract white wool in a Soxhlet extractor with petroleum ether for 2-3 hours to remove fat. Do not further handle the wool, as it may absorb grease from the skin. Squeeze as dry as possible and leave on a steam bath immersed in 5% ammonia solution for an hour to remove petroleum ether, then hang the wool up to dry. Store in a wide-mouth capped jar.

2. 5% ammonia solution. Dilute 5 ml concentrated ammonia to 100 ml with water.

3. Ammonia-solvent mixture. Mix equal proportions of 3N ammonia solution, acetone and ethanol.

PROCEDURE

If necessary, for liquid samples, acidify slightly with acetic acid or $KHSO_4$ (e.g. 30 ml sample + 5 ml 10% $KHSO_4$). If the liquid contains alcohol it should be evaporated first to remove the alcohol and re-diluted. For water-soluble foods such as preserves and sweets, dissolve a suitable weight in 30 ml 5% ammonia in 70% ethanol, leave several hours and centrifuge. For gelatin-based foods, use 5% ammonia in 90% ethanol. In either case, evaporate to a total volume of 30 ml and add 5 ml 10% $KHSO_4$. For high-protein samples soak overnight in 1% ammonia solution, centrifuge and acidify. For high-fat foods such as meat products, de-fat with petroleum ether before applying one of the procedures given above.

For basic dyes, add 10-20 cm of wool to an ammoniacal solution, either separately prepared or at the appropriate stage of one of the extraction procedures above. If the wool absorbs any colour leave it in contact with the warm solution until the colour is transferred to the wool. Remove the wool from the solution, rinse, strip off the dye with 1% acetic acid, make the solution just alkaline with ammonia and dye a fresh piece of wool. Strip again with acetic acid and gently evaporate to a small volume. Carry out appropriate identification tests.

For acidic dyes, add wool to the solution acidified with acetic acid or 10% $KHSO_4$ solution. Wash the wool thoroughly with warm water and then warm with 5 ml ammonia-solvent mixture for about 5 minutes. Remove the wool, gently evaporate the solution to dryness and dissolve the residue in a drop or two of water. Conduct appropriate identification tests.

REFERENCE

Association of Public Analysts. 1960. Separation and Identification of Food Colours Permitted by the Colouring Matters in Food Regulation (1957). London

WATER-SOLUBLE COLOURS
(Polyamide Separation)

PRINCIPLE

The sample is defatted, if necessary, and the colour extracted using a suitable solvent chosen according to the nature of the foodstuff. The colour extract is adsorbed on a polyamide column, washed with various solvents and eluted with a mixture of acetone and ammonia.

APPARATUS

1. Chromatographic tubes, 300 mm, 250 mm x 15 mm and 200 mm x 10 mm, all fitted with a glass stopcock.

2. Oven set at 100-102°C.

3. Water-bath.

4. Soxhlet continuous extraction apparatus and thimbles.

5. Glass evaporating basin.

REAGENTS

1. Acetone.

2. Acetone-ammonia solution: mix 40 ml of acetone, 9 ml of water and 1 ml of ammonia solution (SG = 0.880). This should be freshly prepared.

3. Chloroform.

4. Methanol-ammonia solution: mix 90 ml of methanol, 5 ml of water and 5 ml of ammonia solution.

5. Petroleum ether, boiling range 40-60°C.

6. Acetic acid, glacial.

7. Polyoxyethylene sorbitan mono-oleate solution: mix 1 ml of polyoxyethylene sorbitan mono-oleate ("Tween" 80) with 99 ml of water.

8. Methanol-water-tetramethylammonium hydroxide mixture (40+9+1).

9. Chloroform-absolute ethanol-water-formic acid mixture (100+90+10+1).

10. Hydrochloric acid, 0.5N.

11. Hydrochloric acid, 0.1N.

12. Polyamide staple fibre: nylon 66, 3.3 g per 10,000 m. of fibre obtained from Macherey, Nagel and Co., 5161 Düren, Wertstrasse 628, Federal Republic of Germany.

13. Polyamide powder for column chromatography: MN-CC6 from Macherey, Nagel and Co., grain size, 0.16 mm.

14. Celite 545.

15. Sand, acid washed.

PROCEDURE

For water soluble products (jellies, jams, sweets):

Weigh about 5 g of the sample into a beaker, add 50 ml of water and warm the beaker on a water-bath until the water-soluble material is dissolved. Acidify the mixture with glacial acetic acid.

Place a plug of polyamide staple fibre in the end of the chromatographic tube and add a suspension of polyamide powder in water to the tube to give a column approximately 20 mm high. Rinse the walls of the tube with a small volume of acetone to aid the settling of the polyamide and then place sand on top of the polyamide to form a layer about 6 mm deep.

Pour the hot extract through the column. Wash the column with six 10 ml portions of hot water followed by three 5 ml volumes of acetone (slight air pressure may be used, if necessary). Elute the colours with the minimum volume of acetone-ammonia solution, rejecting the colour-free liquid that is eluted at first. Remove the ammonia by blowing air over the surface of the liquid and then evaporate the solution to about a quarter of the original volume on a waterbath. Add water to give the original eluate volume and adjust the pH to 5-6 with hydrochloric acid 0.5N.

Prepare a polyamide column in a 200 mm chromatographic tube as described above. Add the final solution obtained above to this column and wash with five 5 ml portions of hot water. Elute the dyes with the minimum volume of acetone-ammonia solution. Remove the ammonia in the same way as before and evaporate the solution to near dryness on a steam-bath. Dissolve the residue in a few drops of 0.1N hydrochloric acid and proceed with identification. If erythrosine is suspected, dissolve the residue in water.

For bakery products (cakes, cake powders, pastries):

Weigh about 5 g of the chopped sample into a glass evaporating basin and place the basin in a drying oven at 100° for 30 minutes. Cool and add sufficient petroleum ether to cover the dried sample (about 30 ml) and stir the mixture. Allow the solid to settle and decant the petroleum ether. Repeat this procedure twice more and then allow the residual petroleum ether to evaporate. Grind the sample gently so as to form a coarse powder, add 4 g of Celite 545 and mix.

Place a plug of polyamide staple fibre in the end of a 250 mm chromatography tube and transfer the powdered sample to the tube. Pour 30 ml of acetone onto the top of the column and, when the solvent has percolated the whole length of the column, apply slight air pressure to aid uniform packing. Discard the eluate. Carefully pour 50 ml of the methanol-water-tetramethylammonium hydroxide solution through column. (Slight air pressure may be used if necessary). Adjust the pH of the eluate to approximately 6 by the addition of dilute hydrochloric acid 0.5N. Add 5 ml of the 1% polyoxyethylene sorbitan mono-oleate solution and evaporate the solution to about a quarter of the original volume on a water-bath with the aid of a current of air blown over the surface of the liquid. Add water to give the original eluate volume and allow the solution to cool.

Prepare a polyamide column in a 200 mm chromatographic tube as before. Add the solution of extracted acetone. Wash five times with 5 ml portions of chloroform-absolute ethanol-water-formic acid solution, three times with 5 ml of acetone and finally three times with 10 ml of water. Elute the dyes with the minimum volume of

acetone-ammonia solution, rejecting the eluate until the dyes are eluted. Remove the ammonia in the coloured eluate by blowing a current of air over the surface of the liquid and evaporate the solution to about a quarter of the original volume on a waterbath. Add water to give the original volume of eluate and adjust the pH to about 6 with hydrochloric acid 0.5N.

Add the acidified solution to a polyamide column contained in a 200 mm chromatographic tube. Wash the column with the same volumes of solvents as described in the previous paragraph. Elute the dyes with a minimum volume of acetone-ammonia solution. Remove the ammonia and evaporate to near dryness on a steam-bath. Dissolve the residue in a few drops of 0.1N hydrochloric acid and proceed with appropriate identification. If erythrosine is suspected, dissolve the residue in water.

For meat products:

Weigh 25 g of sample and place on a flat surface, chop up the sample with a knife, add 5 g of acid-washed sand and grind the mixture to a paste. Add 10 g of Celite and mix with a palette knife until a homogeneous mixture is obtained. Transfer the mixture to a thimble, place the thimble in a Soxhlet extractor and extract with chloroform for 2 hours. Remove the sample from the thimble and place it in an evaporating basin to allow the residual chloroform to evaporate.

Place a plug of polyamide staple fibre in the end of a 300 mm chromatographic tube and add the powdered sample to the tube, tapping the column gently to aid packing. Pass methanol-ammonia solution through the column until all of the dyes are eluted. (Slight air pressure may be used if necessary). Add 5 ml of 1% polyoxyethylene sorbitan mono-oleate and evaporate the solution to about a quarter of the original volume on a water-bath, with the aid of a current of air blown over the surface of the liquid. Add water to restore the eluate to its original volume and adjust the pH of the solution to 6 with hydrochloric acid 0.5N.

Prepare a polyamide column in a chromatographic tube in the same way as before. Pour the solution of the dyes through the column. Wash the column three times with 10 ml volumes of water, twice with 5 ml volumes of acetone, twice with 5 ml of a chloroform-absolute ethanol-water-formic acid mixture and twice with 5 ml of acetone. Elute the dyes with the minimum volume of acetone-ammonia solution rejecting the eluate until the dyes are eluted. Remove the ammonia by blowing a current of air over the surface of the liquid and then evaporate the solution to about a quarter of the original volume on a water-bath. Add water to restore the eluate to its original volume and adjust the pH to approximately 6 with 0.5N HCl.

Prepare a polyamide column in a 200 mm chromatographic tube as before. Add the acidified eluate to this column and wash the column as described previously. Elute the dyes with the minimum volume of acetone-ammonia solution. Remove the ammonia by a current of air over the surface of the liquid and evaporate the solution to near dryness. Dissolve the residue in a few drops of 0.1N hydrochloric acid and proceed with appropriate identification. If erythrosine is suspected dissolve the residue in water.

REFERENCE

Lehmann, G., Collet, P., Hahn, H.G. and Ashworth, M.R.F. 1970. Journal of the AOAC 53, 1182.

WATER-SOLUBLE COLOURS
(Quinoline Extraction)

PRINCIPLE

The sample (defatted if necessary) is extracted by quinoline in a buffered medium at pH 3. The quinoline solution is diluted with diethyl ether and extracted successively by water, by ammonia and hydrochloric solutions, and by chloroform. The colouring matters are separated in one or other of these phases, according to their group affinities.

In the case of products manufactured from meat (the protein constituents of which are likely to absorb the colouring matters to a high degree) extraction by quinoline is preceded by treatment using a liquid ion-exchange resin (Amberlite LA 2).

APPARATUS

1. Water bath.
2. Homogenizer.
3. Centrifuge with ground-glass stoppered tubes of volume 50 ml, height not less than 20 cm and external diameter about 2 cm.
4. Separating funnels.
5. Specimen tubes, wide-necked, volume approximately 7 ml.
6. Pestle and mortar.
7. Oven, set at 100 - 102°C.

REAGENTS

1. Buffer solution (pH 3): Dissolve in distilled water 2 g of sodium acetate ($CH_3COONa.3H_2O$), add 25 ml of glacial acetic acid and dilute to 1 litre with distilled water.
2. Quinoline (a light yellow colour does not prohibit its use).
3. Strong ammonia solution.
4. Diethyl ether (free from peroxides).
5. Ammonia, 10% solution: Dilute 40 ml concentrated ammonia to 100 ml with water.
6. Hydrochloric acid, 1N.
7. Chloroform.
8. Ethanol, 96%.
9. Ethanol, 70% (v/v).
10. Acetic acid, glacial.
11. Acetone.
12. Sea sand (silver sand) washed in hydrochloric acid and calcined.

13. "Celite" in powder form.

14. Amberlite LA 2 (BDH, Rohm and Haas, or equivalent).

PROCEDURE

The amounts of sample, auxiliary substances and solvents may be modified depending on the nature of the sample and the intensity of its colouring.

For foods with a high sugar content, alcoholic drinks and dairy products:

Homogenize solid foods, take 10 g and mix thoroughly with 40 ml of buffer solution (pH 3). Place 30 ml of this mixture into a centrifuge tube. For liquid foods, place 10 ml of the sample into a centrifuge tube and add 20 ml of buffer solution.

Next add 10 ml of quinoline, shake vigorously, then centrifuge for 5 minutes at 1200 rpm. Withdraw and discard the aqueous solution as completely as possible by suction using a Pasteur pipette. Shake the quinoline phase with 20 ml of water, centrifuge and reject the upper aqueous phase. Repeat this washing if the quinoline phase is cloudy. Filter using a dry filter-paper and collect the filtrate in another centrifuge tube.

Add to the quinoline solution 30 ml of diethyl ether and 1 ml of water. Shake vigorously and centrifuge. Repeat extractions with 1 ml aliquots of water until no colour is visible in the aqueous phase. Combine all coloured aqueous extracts in a specimen tube and retain the quinoline-ether solution.

If the combined aqueous extract is coloured, shake it several times with a little diethyl ether. Discard the ether after separation of the phase, then make the solution just acid with acetic acid and evaporate on a water bath until the volume is reduced to about 0.5 ml. Proceed with identification.

Shake the ether/quinoline solution with 10 ml of hydrochloric acid 1N. Colouring of the acid aqueous phase indicates the presence of basic colours. Such colouring is red in the case of rhodamine B. Centrifuge, remove the ether quinoline phase as completely as possible and discard it. Shake the coloured acid aqueous solution with 2 ml of chloroform, allow to separate, centrifuging if necessary, and discard the aqueous phase. Wash the chloroform solution twice with 1 ml of water, discard the aqueous phase after each wash and treat the chloroform solution as the aqueous extract above.

If the ether/quinoline phase remains coloured (which may indicate the presence of erythrosine, eosine, phloxine and/or certain natural colourings) add 1 ml of ammonia and shake vigorously. Repeat the extractions with 2 ml aliquots of 10% ammonia until no colour is visible in the aqueous phase. Combine all of the coloured ammonia extracts in a specimen tube and treat the same as the aqueous extract, above.

Dilution with ether converts certain colours into a colourless form which remains in solution in the ether/quinoline. If rhodamine B is the only red colouring matter present in the sample, the loss in colour of the quinoline phase on dilution with ether confirms that this colour is present.

For food with a high starch or protein content (with the exception of products manufactured from meat, or containing egg yolks):

If the food has a high fat content, remove the fat as follows: Put 10 g of the sample into a mortar. Grind with 3 g of silver sand, 6-7 g of "Celite" and 30 to 40 ml of acetone. Decant the liquid and filter through a fluted filter paper. Return the residue to the mortar, triturate with 30-40 ml of acetone, decant and filter as before. Repeat twice more. Leave the defatted residue to dry at room temperature and then complete drying in the oven for 30 minutes. Grind the residue to a powder. For low fat foods dry if necessary and grind to a powder.

Put 2 g of the dried, pulverized food in a mortar, add 15 ml of quinoline which has been previously saturated with water. Extract the colouring matter as completely as possible by grinding the sample with quinoline. Add 20 ml of buffer solution and grind again. Transfer the mixture to a centrifuge tube and shake vigorously. Centrifuge for 10 minutes at 1200 rpm and reject the upper aqueous phase. If an emulsion forms at the level between the phases, it should be drawn off with the aqueous phase. Add 20 ml of water, shake vigorously, centrifuge for 10 minutes at 1200 rpm. Discard the aqueous phase as completely as possible by use of suction and filter the cake and quinoline under vacuum through a sintered glass crucible of porosity G3 into a centrifuge tube. Pass 5 ml of water through the filter and add a further 5 ml to the total filtrate, shake the quinoline and water vigorously and centrifuge. Draw off the lower quinoline layer and filter it through a dry filter paper into a centrifuge tube.

Add 30 ml of diethyl ether and 1 ml of water to the quinoline filtrate, shake vigorously and centrifuge. If colour is present in the aqueous phase transfer it to a fresh centrifuge tube. Repeat extractions with 1 ml aliquots of water until no colour is visible in the aqueous extracts. Add 10 ml of quinoline and 20 ml of buffer solution pH 3 to the combined coloured aqueous extracts, shake vigorously and centrifuge. Draw off the lower quinoline layer and transfer it to a centrifuge tube. Add 30 ml of diethyl ether. Extract the colours into water or 10% ammonia respectively. Wash separately the combined water and combined ammonia extracts as described above for high sugar foods.

Preserved fruits and fruits in syrup:

Remove the surface sugar from the fruits with a minimum of water. Break down the sample into small pieces, take 10 g, add 100 ml of ethanol 70% and heat in a water bath until the colouring dissolves. Decant the liquid and evaporate the ethanol to a volume of about 10 ml. Transfer to a centrifuge tube using 20 ml of buffer solution, add 10 ml of quinoline and then proceed as above for high sugar foods.

Meat products:

Chop finely 10 g of sample. Defat the sample as described above using petroleum ether in place of acetone. Grind the dried defatted meat with a solution of Amberlite LA2 (1.25 ml) in quinoline (15 ml). Add 5 ml of water and grind; leave for 1 hour, grinding occasionally.

If some supernatant liquid is apparent decant it through a cotton-wool plug into a centrifuge tube. Grind the residue with 4 x 5 ml aliquots of quinoline, decanting the supernatant liquid after each washing through the cotton-wool plug. Centrifuge the residue (2500 rpm) and filter the supernatant liquid through the cotton-wool plug.

If the ground mixture is a paste, (e.g. as with luncheon meat) with no supernatant liquid, grind with 15 ml of quinoline. Transfer the mixture to a centrifuge tube and centrifuge (2500 rpm). Filter the supernatant liquid through a cotton-wool plug. Wash out the mortar with 5 ml of quinoline into the centrifuge tube. Stir the contents of the tube and centrifuge (2500 rpm). Filter through the cotton-wool plug.

Add 20 ml of buffer pH 3 to the combined filtrates from either of the above, shake and centrifuge. Draw off the quinoline and filter through cotton-wool into a tapered centrifuge tube. Add 30 ml of diethyl ether + 2 ml of ammonia 10%; shake and centrifuge. Transfer the aqueous phase to another centrifuge tube. Repeat the extractions with ammonia until no colour is visible in the aqueous phase. To the combined ammonia extracts add 20 ml of buffer + 10 ml of quinoline; shake and centrifuge. Draw off the quinoline and filter through cotton-wool into a tapered centrifuge tube. Add 30 ml of diethyl ether and extract with 2 ml aliquots of 10% ammonia, shaking and centrifuging each time, until the final ammonia extract contains no colour. Combine the coloured ammonia extracts in a specimen tube and treat as described for aqueous extracts, under high sugar foods.

REFERENCE

Mottier, M. and Potterat, M., 1953. Travaux de Chimie Alimentaire et d'Hygiene 44, 293.

WATER-SOLUBLE COLOURS
(Thin-Layer Chromatography)

PRINCIPLE

The extracted dye is identified by thin-layer chromatography using cellulose and silica gel. The dyes are grouped according to a code depending on their R_f values in different solvents. This is of assistance in identifying some of the dyes not usually encountered in permitted lists.

APPARATUS

1. Thin-layer chromatographic apparatus, including spreaders for the preparation of thin layers 0.25 mm thick on 200 x 200 mm glass plates, chromatographic development tanks and 5 µl pipettes.

REAGENTS

1. Cellulose powder. Microcrystalline cellulose (available from Applied Science Laboratories Inc. or equivalent). Prepare plates as follows: shake 20 g cellulose powder with 60 ml methanol for 3 minutes and blend at high speed for 30 seconds. spread onto plates and dry in an oven at 80°C. Alternatively, use prepared plates as purchased.

2. Reference dye solutions. 0.1% in water.

3. Chromatographic solvents. All solvent mixtures should be freshly prepared. The following solvent mixtures may be used with cellulose plates:

   a. Trisodium citrate (2g), water (85 ml), ammonia (15 ml).

   b. t-Butanol:propanoic acid:water (50:12:3).

   c. Trisodium citrate (2g), hexamine (5g), water (50 ml).

   d. 2-Methylpropan-1-ol:water:ethanol:ammonia (25:25:50:2).

   e. Propan-1-ol:ethyl acetate:water (6:1:3).

   f. Butan-1-ol:water:glacial acetic acid (20:12:10).

   g. Hydrochloric acid:water (23:77).

   h. Butan-1-ol:water:pyridine:ethanol (4:5:2:2).

   i. Ethylmethylketone:acetone:water:ammonia (70:30:30:0.5).

   j. Butan-1-ol:water:ethanol:quinoline (4:5:3:2).

The following solvent mixtures may be used with silica gel plates:

   k. Propan-2-ol:ammonia (4:1).

   l. Propan-2-ol:ammonia (85:15).

   m. Methanol:chloroform:water:quinoline (4:2:2:2).

   n. Methanol:chloroform:quinoline (4:4:2).

   o. Propan-2-ol:chloroform:water:diethylamine (50:25:20:15).

PROCEDURE

Place 2 spots of 1-2 µl of the dye solution onto each of four cellulose plates at a distance of at least 20 mm from the edge and bottom of the plate. Also spot on the plates 1-2 µl of a solution of Orange G and a solution of Amaranth as reference spots and place a spot of a mixture of Orange G and Amaranth on top of one of the sample spots. Dry the spots by placing the plates in an oven at 105°C for 5-10 minutes. Develop the cooled plates in solvents a, b, c and d for a length of run of about 150 mm at room temperature. Remove the plates from the tanks and allow them to air dry. When the plates are dry rule lines across so as to divide the plates into the following sections: code A: spots travelling above Orange G; code B: spots travelling with Orange G; code C: spots travelling below Orange G but above Amaranth; code D: spots travelling with Amaranth; code E: spots travelling below Amaranth.

Check whether the sample has affected the development characteristics of Orange G and Amaranth and if so make allowance for this when dividing the plate into sections. Observe which section the spots from the sample solution appear in for each plate and write down all possible composite codes for each spot by listing the code individual letters in the order - solvent a, solvent b, solvent c, solvent d. Compare the codes with the list given in Table I and hence obtain a preliminary identification of the dyes. When two or more spots are similar in colour, cross code the dyes so that all possible dyes are obtained from Table I. Also if a dye is visible in one solvent but not in another then this indicates that the dye is masked by another dye and so all codes for spots in that solvent must be used in constructing the composite codes. A further identification of the dyes may be obtained by calculating the R_f and R_x (with respect to Orange G) values and referring to the Tables II-V. This will eliminate some of the dyes obtained from Table I. All R_f and R_x values are calculated by measuring to the leading edge of the spots.

Confirm identity of the sample dye by chromatography on a plate with standard spots of the suspected colours using suitable solvents. Also overspot spots of the sample solution with spots of the suspected dyes. The unknown dye is identified by giving a single spot with the correct standard while all the other standards should give rise to double spots. If the sample contains several dyes, more than one solvent may be necessary for complete confirmation of the identity of the dyes.

INTERPRETATION

In compiling the table of codes for the dyes, slight variations in the development characteristics of the dyes have been taken into account so that some dyes occur under a number of different codes. Brown FK, Chocolate Brown FB and Chocolate Brown HT have not been included in this table as they streak in the solvents used. If the standard dyes, Orange G and Amaranth, act differently when over-spotted on the sample from when they are spotted separately on the plate, then the spots in the sample should be coded twice, once using the standards in the sample to divide up the plate and once using the standard spotted separately to divide up the plate. By this means all possible dyes will be included but a number of these will be rejected on the basis of colour and R_f value. However, do not discount dyes which could give rise to the colour of the spot (e.g. an orange coloured spot may be a red and yellow dye superimposed).

As most problems arise from the possibility of a red and yellow dye being together in the mixture, the separation of the reds, oranges and yellows are set out in Table VI. All R_f and R_x values have been calculated by measuring to the leading edge of a spot as this was found to be more reliable for spots

which tail. When confirming the identity of a dye by running it with standard dyes it is useful to observe the plate under UV light of 254 nm and 350 nm as some of the dyes fluoresce.

The following mixtures of dyes could not be separated in any of the solvents tried: Chocolate Brown HT and Chocolate Brown FB, Ponceau 3R and Ponceau MX, Violet 5BN and Violet BNP. Chocolate Brown HT can be tentatively distinguished from Chocolate Brown FB by running in solvent o, on silica gel. Chocolate Brown HT produces two spots and a streak from the spotting line. The two spots travel higher than the streak from Chocolate Brown FB.

Co-extractives may affect the running characteristics of various dyes but by over-spotting the sample with the suspected dyes in the final confirmation any irregularities should not affect the identification of the dyestuff.

REFERENCE

Hoodless, R.A., Thomson, J. and Arnold, J.E. 1971. Journal of Chromatography 56, 332.

Table I

(Codes for colours chromatographed using solvents a, b, c and d).

Code	Possible Colour
AAAA	Blue VRS
	Brilliant Blue
	Light Green Yellowish
AAAB	Brilliant Blue
	Fast Green
	Green S
	Light Green Yellowish
	Patent Blue V
	Yellow 2G
AAAC	Yellow 2G
AABA	Scarlet GN
AACA	Scarlet GN
ABAA	Brilliant Blue
	Light Green Yellowish
ABAB	Brilliant Blue
	Light Green Yellowish
ABAC	Acid Magenta
ABAD	Acid Magenta
ACAC	Acid Magenta
ACAD	Acid Magenta
ACCD	Tartrazine
ADCD	Ponceau 6R
	Tartrazine
ADCE	Ponceau 6R
AECD	Ponceau 6R
AECE	Ponceau 6R
BAAA	Guinea Green B
	Violet 5BN
	Violet BNP
BBBB	Orange G
BCBC	Ponceau 4R
BCCC	Ponceau 4R
BCCD	Tartrazine
BDCD	Tartrazine
CAAA	Guinea Green B
	Violet BNP
	Violet 5BN
	Violet 6B
CACB	Chrysoin S
CACC	Chrysoin S
CBCA	Naphthol Yellow S
CBCB	Orange GGN
	Sunset Yellow
CCBC	Ponceau 4R
CCCB	Acid Yellow
	Orange GGN
	Sunset Yellow
CCCC	Acid Yellow
	Orange GGN
	Ponceau 4R
	Red 2G
	Sunset Yellow
CCCD	Acid Yellow
CCDC	Red 2G
DAAA	Rhodamine B
	Violet 6B
DABA	Rhodamine B
DACA	Orange RN
DACB	Chrysoin S
DACC	Chrysoin S
	Orange RN
DADA	Orange RN
DADC	Orange RN
DBCA	Naphthol Yellow
DBCB	Orange GGN
	Sunset Yellow
DCCA	Orange RN
DCCB	Orange GGN
	Sunset Yellow
DCCC	Acid Yellow
	Orange GGN
	Orange RN
	Sunset Yellow
DCCD	Acid Yellow
	Orange GGN
	Sunset Yellow
DCDA	Orange RN
DCDC	Orange RN
DCDD	Red 6B
DCDE	Red 6B
DCED	Red 6B
DCEE	Red 6B
DDDD	Amaranth
	Red 6B
DDDE	Red 6B
DDEE	Red 6B
DEDD	Red 6B
DEDE	Red 6B
DEED	Red 6B
DEEE	Red 6B
EAAA	Auramine
	Methyl Violet
	Rhodamine B
	Violet 6B
EABA	Auramine
	Methyl Violet
	Rhodamine B
EACA	Auramine
	Eosine
	Erythrosine
	Chrysoidine
	Orange I
	Orange RN
EACB	Chrysoidine
	Eosine
	Erythrosine
	Orange I
EACC	Chrysoin S
	Orange RN
EADB	Orange I
	Quinoline
	Yellow
EADC	Orange RN
	Quinoline
	Yellow
EAEB	Quinoline
	Yellow
EAEC	Quinoline
	Yellow
EBCB	Fast Red E
EBCC	Fast Red E
EBCD	Carmoisine
EBCE	Carmoisine
EBDB	Fast Red E
	Quinoline
	Yellow
EBDC	Fast Red E
	Quinoline
	Yellow
EBDD	Carmoisine
EBDE	Carmoisine
EBEB	Quinoline
	Yellow
EBEC	Quinoline
	Yellow
ECCB	Fast Red E
ECCC	Fast Red E
	Indigo Carmine
	Orange RN
	Red 10B

Table 1 (continued)

Code	Possible Colour	Code	Possible Colour	Code	Possible Colour
ECCD	Carmoisine	ECDE	Carmoisine	EDDE	Red 6B
	Indigo Carmine		Red 6B		Red 10B
	Red 10B		Red 10B	EDED	Black 7984
ECCE	Carmoisine	ECEC	Bordeaux B		Black PN
	Red 10B		Ponceau 3R		Red 6B
ECDA	Orange RN		Ponceau MX	EDEE	Black 7984
ECDB	Fast Red E		Ponceau SX		Black PN
ECDC	Bordeaux B	ECED	Ponceau SX		Red 6B
	Fast Red E		Red 6B		Red FB
	Indigo Carmine	ECEE	Red 6B	EEDD	Red 6B
	Orange RN	EDCC	Indigo Carmine	EEDE	Red 6B
	Ponceau 3R		Red 10B	EEED	Black 7984
	Ponceau MX	EDCD	Indigo Carmine		Black PN
	Ponceau SX		Red 10B		Red 6B
	Red 10B	EDCE	Red 10B	EEEE	Black 7984
ECDD	Carmoisine	EDDC	Indigo Carmine		Black PN
	Indigo Carmine		Red 10B		Red 6B
	Ponceau SX	EDDD	Indigo Catmine		Red FB
	Red 6B		Red 6B		Indanthrene
	Red 10B		Red 10B		Blue

Table II

(R_f and R_x with respect to Orange G values for red colours).

Colour	Colour Index Number	Solvent	Approximate R_f values									Solvent	Approximate R_x values								
			a	b	c	d	e	f	h	i	k	a	b	c	d	e	f	h	i	k
Amaranth	46185		0.6	0.3	0.5	0.6	0.4	0.2	0.6	0.4	0.4		0.8	0.4	0.5	0.8	0.7	0.4	0.8	0.4	0.9
Bordeaux B	16180		0.2	0.6	0.4	0.6	0.5	0.6	0.7	1.0	0.4		0.3	0.9	0.4	0.8	0.9	1.0	1.0	1.0	0.9
Carmoisine	14720		0.3	0.7	0.6	0.5	0.7	0.6	0.8	0.9	0.4		0.4	1.1	0.6	0.7	1.1	1.0	1.1	0.9	0.9
Eosine	45380		0.2	1.0	0.7	0.8	1.0	1.0	0.9	1.0	0.6		0.3	1.5	0.7	1.1	1.6	1.7	1.3	1.0	1.4
Erythrosine	45430		0.1	1.0	0.7	0.9	1.0	1.0	0.9	1.0	0.7		0.2	1.5	0.7	1.2	1.6	1.7	1.3	1.0	1.6
Fast Red E	16045		0.4	0.7	0.6	0.7	0.6	0.5	0.8	1.0	0.4		0.6	1.0	0.6	1.0	1.0	0.9	1.1	1.0	0.9
Ponceau 3R	16155		0.2	0.6	0.4	0.6	0.5	0.5	0.8	0.9	0.3		0.3	0.9	0.4	0.8	0.7	0.9	1.1	0.9	0.7
Ponceau 4R	16255		0.7	0.5	0.9	0.6	0.4	0.3	0.7	0.6	0.2		1.0	0.6	1.0	0.8	0.7	0.5	1.0	0.6	0.4
Ponceau 6R	16290		0.8	0.2	0.8	0.4	0.3	0.1	0.6	0.2	0.1		1.1	0.2	0.8	0.5	0.4	0.2	0.8	0.2	0.2
Ponceau MX	16150		0.2	0.7	0.5	0.6	0.5	0.5	0.8	0.9	0.4		0.3	0.9	0.5	0.8	0.9	0.9	1.1	0.9	0.8
Ponceau SX	14700		0.4	0.7	0.5	0.6	0.5	0.5	0.8	0.9	0.4		0.6	0.9	0.5	0.8	0.9	0.9	1.1	0.9	0.8
Red 2G	18050		0.6	0.6	0.7	0.6	0.4	0.5	0.7	0.9	0.4		0.8	0.8	0.7	0.8	0.7	0.9	1.0	0.9	0.9
Red 6B	18055		0.4	0.3	0.4	0.5	0.4	0.2	0.6	0.5	0.4		0.6	0.4	0.4	0.7	0.7	0.4	0.8	0.5	0.9
Red 10B	17200		0.2	0.5	0.6	0.6	0.4	0.3	0.7	0.8	0.4		0.3	0.6	0.6	0.8	0.7	0.5	1.0	0.8	0.9
Red FB	14780		0.0	0.3	0.1	0.2	0.4	0.2	0.7	0.4	0.6		0.0	0.4	0.1	0.3	0.7	0.4	1.0	0.4	1.3
Rhodamine B	45170		0.5	1.0	0.9	1.0	1.0	1.0	0.9	1.0	0.8		0.7	1.5	1.0	1.3	1.6	1.7	1.4	1.0	1.8
Scarlet GN	14815		0.9	0.7	0.9	0.8	0.8	0.6	0.8	1.0	0.5		1.1	0.9	1.0	1.1	1.2	1.0	1.1	1.0	1.2

Table III

(R_f and R_x with respect to Orange G values for yellow and orange colours.)

Colour	Colour Index Number	Approximate R_f values									Approximate R_x value								
		Solvent									Solvent								
		a	b	c	d	e	f	g	h	k	a	b	c	d	e	f	g	h	k
Auramine	41000	0.3	1.0	0.9	1.0	0.9	1.0	-	0.8	0.8	0.4	1.4	1.0	1.6	1.4	1.9	-	1.3	1.8
Acid Yellow	13015	0.7	0.6	0.9	0.6	0.6	0.5	0.7	0.7	0.4	0.8	0.9	1.0	0.9	0.9	0.9	1.1	1.0	1.0
Chrysoidine	11270	0.1	0.8	0.7	0.9	0.8	0.9	0.1	0.9	0.8	0.2	1.2	0.7	1.6	1.2	1.7	0.2	1.4	1.8
Chrysoin S	14270	0.5	0.8	0.8	0.6	0.9	0.7	0.4	0.8	0.5	0.6	1.2	0.9	0.9	1.3	1.4	0.7	1.2	1.0
Orange G	16230	0.8	0.7	0.9	0.6	0.7	0.5	0.7	0.7	0.4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Orange GGN	15980	0.6	0.7	0.8	0.6	0.6	0.5	0.2	0.7	0.4	0.8	1.0	0.9	1.0	1.0	1.0	0.3	1.0	1.0
Orange I	14600	0.4	0.8	0.8	0.7	0.9	0.7	0.1	0.8	0.5	0.6	1.2	0.9	1.1	1.3	1.4	0.2	1.2	1.2
Orange RN	15970	0.4	0.9	0.7	0.6	0.6	0.5	0.1	0.7	0.7	0.5	1.2	0.8	0.9	0.8	0.9	0.2	1.0	1.6
		0.5			0.8	0.9	0.8		0.8		0.7			1.3	1.4	1.5		1.2	
Quinoline Yellow	47005	0.1	0.7	0.4	0.6	0.7	0.6	0.1	0.6	0.7	0.1	1.0	0.5	1.0	1.1	1.2	0.1	0.9	1.6
		0.3							0.7		0.4							1.0	
Sunset Yellow	15985	0.6	0.7	0.8	0.6	0.6	0.5	0.2	0.7	0.4	0.7	0.9	0.9	1.0	0.9	1.0	0.4	1.0	1.0
Tartrazine	19140	0.8	0.4	0.8	0.4	0.5	0.3	0.4	0.5	0.3	2.0	0.6	0.8	0.6	0.7	0.6	0.6	0.7	0.6
Yellow 2G	18965	0.9	0.8	1.0	0.6	0.8	0.6	0.9	0.7	0.4	1.1	1.2	1.1	1.0	1.2	1.2	1.5	1.0	1.0

Table IV

(R_f and R_x with respect to Orange G values for brown and violet colours.)

Colour	Colour Index Number	Approximate R_f values								Approximate R_x value							
		Solvent								Solvent							
		a	b	c	d	e	k	n	o	a	b	c	d	e	k	n	o
Brown FK	N/A	S	S	S	0.6	S	0.7	S	0.4	S	S	S	0.9	S	1.1	S	0.6
							0.8	S	0.5						1.3	S	0.7
Chocolate Brown FB	N/A	S	S	S	S	S	0.0	0.0	S	S	S	S	S	S	0.0	0.0	S
Chocolate Brown HT	20285	S	S	S	S	S	0.0	0.0	S	S	S	S	S	S	0.0	0.0	S
Acid Magenta	42685	1.0	0.4	0.9	0.6	0.5	0.2	-	-	1.4	0.7	1.1	0.8	0.8	0.3	-	-
						0.6					1.0			0.9			
Methyl Violet	42535	S	1.0	0.8	1.0	1.0	0.8	0.8	0.7	S	1.5	1.0	1.4	1.6	1.3	1.9	1.9
				0.9			0.9					1.1			1.4		
Violet BNP		0.7	0.8	0.9	0.9	0.9	0.5	0.4	0.5	1.0	1.3	1.1	1.3	1.4	0.7	1.1	0.7
Violet 5BN	42650	0.7	0.8	0.9	0.9	0.9	0.5	0.4	0.5	1.0	1.3	1.1	1.3	1.4	0.7	1.1	0.7
Violet 6B	42640	0.6	0.8	1.0	1.0	0.9	0.5	0.4	0.6	0.8	1.3	1.2	1.4	1.4	0.8	1.1	0.9
							0.6	0.5							0.9	1.2	

Note: S = streak on plate.

Table V

(R_f and R_x with respect to Orange G values for green, blue and black colours.)

Colour	Colour Index Number	Solvent	Approximate R_f values									Solvent	Approximate R_x values								
			a	b	c	d	e	k	l	m	j		a	b	c	d	e	k	l	m	j
Fast Green FC	42053		0.9	0.8	1.0	0.8	0.8	0.1	0.1	0.8	0.8		1.2	1.1	1.1	1.0	1.1	0.5	0.1	1.0	1.0
Green S	44090		0.9	0.8	0.9	0.8	0.8	0.1	0.1	0.7	0.8		1.2	1.1	1.0	1.0	1.2	0.4	0.2	0.9	1.0
Guinea Green B	42085		0.7	0.9	1.0	1.0	0.9	0.3	0.3	0.8	0.9		0.9	1.2	1.1	1.3	1.3	1.1	1.4	1.0	1.2
Light Green Yellowish	42095		0.9	0.8	1.0	0.9	0.8	0.3	0.1	0.8	0.8		1.1	1.1	1.1	1.2	1.1	0.9	0.5	1.0	1.0
Blue VRS	42045		0.9	0.9	1.0	0.9	0.9	0.3	0.2	0.8	0.9		1.1	1.2	1.1	1.2	1.3	1.1	1.0	1.0	1.2
Brilliant Blue FCF	42090		0.9	0.8	1.0	0.9	0.8	0.3	0.2	0.8	0.8		1.1	1.1	1.1	1.2	1.1	1.0	0.7	1.0	1.0
Indigo	73015		0.2	0.4	0.5	0.6	0.5	0.3	0.2	0.8	0.7		0.3	0.6	0.6	0.8	0.7	1.0	1.0	1.0	0.9
Carmine			0.3										0.4								
Patent BlueV	42051		0.9	0.9	1.0	0.9	0.9	0.1	0.0	0.8	0.9		1.2	1.2	1.1	1.1	1.3	0.2	0.0	1.0	1.2
Black 7984	27755		0.2	0.3	0.2	0.4	0.4	0.1	0.0	0.7	0.5		0.2	0.4	0.2	0.6	0.5	0.4	0.0	0.9	0.7
Black PN	28440		0.4	0.3	0.2	0.4	0.4	0.1	0.0	0.7	0.5		0.4	0.4	0.2	0.6	0.5	0.4	0.0	0.9	0.9

Table VI

(Separation of reds, oranges and yellows using four solvent mixtures.) (The listing is in order of decreasing R_f. Those colours in brackets do not completely separate from each other.)

Solvent 3a	Solvent 3b	Solvent 3c	Solvent 3d
Yellow 2G	[Eosine	Yellow 2G	[Rhodamine B
Scarlet GN	Erythrosine	Rhodamine B	[Auramine
Tartrazine	[Rhodamine B	Auramine	[Chrysoidine
Ponceau 6R	Auramine	Orange G	[Orange RN
Orange G	Orange RN	[Scarlet GN	Erthyrhrosine
Ponceau R4	[Chrysoin S	Acid Yellow	[Eosine
[Acid Yellow	Chrysoidine	Chrysoin S	Scarlet GN
Red 2G	[Orange I	Naphthol	Naphthol
Orange GGN	Yellow 2G	[Yellow S	Yellow S
Sunset Yellow	[Scarlet GN	[Eosine	[Orange I
Naphthol	Naphthol	Erythrosine	[Orange G
[Yellow S	[Yellow S	Ponceau 4R	[Yellow 2G
[Orange RN	[Orange G	Ponceau 6R	[Fast Red E
Amaranth	Carmoisine	Orange GGN	Acid Yellow
[Chrysoin S	Quinoline	Orange I	Chrysoin S
[Orange I	[Yellow	Orange PN	Orange GGN
Rhodamine B	[Fast Red E	Sunset Yellow	Quinoline
Red 6B	Orange GGN	[Tartrazine	Yellow
Ponceau SX	[Sunset Yellow	[Amaranth	[Sunset Yellow
[Fast Red E	[Bordeaux B	Carmoisine	[Bordeaux B
[Carmoisine	Ponceau 3R	Fast Red E	Ponceau 3R
Bordeaux B	Ponceau MX	Ponceau SX	Ponceau 4R
Auramine	Ponceau SX	Red 2G	Ponceau MX
Quinoline	Acid Yellow	Red 10 B	Ponceau SX
[Yellow	[Orange RN	[Chrysoidine	Red 2G
[Eosine	[Ponceau 4R	[Bordeaux B	Red to B
Ponceau 3R	[Red 2G	Ponceau 3R	[Orange RN
Ponceau MX	[Red 10 B	Ponceau MX	[Amaranth
Red 10 B	[Tartrazine	Quinoline	Carmoisine
[Erythrosine	[Amaranth	[Yellow	[Tartrazine
[Chrysoidine	Ponceau 6R	Red 6B	[Ponceau 6R
Quinoline	Red 6B	Red FB	[Red 6B
[Yellow	[Red FB		Red FB
Red FB			

WATER-SOLUBLE COLOURS
(Paper Chromatography)

PRINCIPLE

The extracted colours are identified by paper chromatography and comparison with standards. Identity is confirmed for some dyes by reducing the dye on the paper with titanous chloride solution. This splits the molecule at the azo-group.

APPARATUS

1. Development tank for paper chromatography suitable for holding sheets of paper 200 x 260 mm.
2. Paper filter sheets for chromatography, 200 x 260 mm.
3. Microcapillary pipettes, 2 microlitres.
4. Filter papers, diameter 7 cm coarse grain, e.g. Whatman No. 4.
5. Absorbent cotton-wool.
6. Sintered glass crucible, porosity G3.

REAGENTS

1. Reference colours.
2. Elution solvents (prepare as required):
   a. Hydrated tri-sodium citrate (2g):Ammonia (20ml):water (80ml).
   b. 2.5% (w/v) hydrated trisodium citrate:colourless pyridine (20:1).
   c. n-Butanol:ethanol:water:ammonia (100:21:42:2).
   d. n-Butanol:ethanol:water:ammonia (50:25:25:10) (for descending chromatography, saturate the chromatography tank in advance for 24 hours).
   e. n-Butanol:ethanol:water (50:26:24).
   f. n-Butanol:water:colourless pyridine:ethanol (40:40:20:10) (for descending chromatography, saturate the chromatography tank in advance for 24 hours).
   g. t-Butanol:0.4% (w/v) potassium chloride:propionic acid (50:50:240).
3. Sulphanilic acid (4-aminobenzenesulphonic acid).
4. Naphthionic acid (4-aminonaphthalene-1-sulphonic acid).
5. Metanilic acid (3-aminobenzenesulphonic acid).
6. 2,4-Dimethyl-5-aminobenzenesulphonic acid.
7. 2,5-Diaminobenzenesulphonic acid.
8. Solution of 15 percent (w/v) titanium trichloride.

9. n-Propanol.

10. Ammonia solution, with 25 percent (w/v) of NH_3.

11. p-Dimethylaminobenzaldehyde Reagent: dissolve 1 g of p-dimethylaminobenzaldehyde in 20 ml of ethanol 96%, add 180 ml of n-butanol and 30 ml of concentrated hydrochloric acid.

12. Petroleum ether, boiling range 40-60°C.

PROCEDURE

Spot three chromatography papers with the unknown colour extract. Also spot appropriate reference colours. Develop each by ascending chromatography using elution solvents a or b for the first paper, c or d for the second and either e, f or g for the third paper.

With the exception of orange GGN, sunset yellow FCF and Ponceau SX, a colour isolated from a sample and a reference colour are considered as identical if their chromatographic behaviours (R_f value, colour, shade) is the same after elution by three elution solvents which comply with the conditions mentioned above. When the colours orange GGN, sunset yellow FCF and Ponceau SX are present, identification must be confirmed by the supplementary procedure described below.

Spot a chromatography paper with the solutions of the colour to be identified, the reference colour and sulphanilic acid, naphthionic acid, metanilic acid, 2,4-dimethyl-5-aminobenzenesulphonic acid and 2,5-diaminobenzenesulphonic acid.

Dry the spots using a current of warm air. Overspot the dry spots with 6 microlitres of a mixture of 2 ml of titanium trichloride solution and 9 ml of distilled water. In most cases this treatment results in decolorization of the stains. If this is not the case, repeat this treatment until decolorization has been completed. Dry the stains thus treated with a current of warm air.

Develop by descending chromatography at 15-20°C, for at least 16 hours, using a mixture of n-propanol (2 parts by volume) and ammonia (1 part by volume). Dry the chromatogram in air. Spray with a solution of p-dimethylaminobenzaldehyde. Compare the reactions given by the colours to be identified with those of the reference colours and sulphonic acids. Orange GGN gives a yellow stain with the same R_f values as metanilic acid, sunset yellow FCF gives a yellow stain with the same R_f value as sulphanilic acid and Ponceau SX gives a yellow stain with the same R_f value as 2,4-dimethyl-5-amino-benzenesulphonic acid.

Under the same operating conditions, the following reactions are obtained from other colours: Yellow stain with the same R_f value as naphthionic acid: Fast red E, azorubine, amaranth, cochineal red A, Ponceau 6R. Yellow stain with the same R_f value as sulphanilic acid: Acid yellow G, chrysoin, tartrazine, brilliant black BN. Red stain with the same R_f value as 2,5-diaminobenzenesulphonic acid: Acid yellow G.

REFERENCE

EEC Document 1867/VI/72/F/Rev.1.

OIL-SOLUBLE COLOURS
(Isolation and Identification)

PRINCIPLE

The food fat (in the unaltered state or extracted from the food) is dissolved in petroleum ether. The solution is subjected to chromatography on a column of aluminium oxide, and the colouring matters undergo elution by means of several elution solvents. The eluates are evaporated to dryness under vacuum and the residues (subjected to saponification if need be) are taken up in diethyl ether and identified by thin-layer chromatography, benzene being used as elution solvent. Adequate safety precautions must be taken when using benzene. Toluene is an alternate solvent but use must be validated.

APPARATUS

1. Balance.

2. Aluminium dish of low form, diameter 7 cm.

3. Drying chamber, set at 60°C.

4. Filter papers, diameter 7 cm.

5. Filter papers, coarse, in sheets.

6. Soxhlet apparatus, with accessories.

7. Water bath.

8. Graduated test-tubes, 10 ml, 25 ml, 50 ml, 100 ml and 250 ml.

9. Beakers, 50 ml.

10. Chromatography tube 20 cm x 1 cm diameter with a tap.

11. Absorbent cotton-wool.

12. Volumetric pipette, 2 ml.

13. Round-bottom flask, 100 ml, with standard ground-glass joint.

14. Rotary evaporator.

15. Bunsen burner or electrically heated plate.

16. Condenser.

17. Separating funnels, 250 ml.

18. Development tank for TLC capable of holding plates 200 x 200 cm.

19. TLC plates 200 x 200 cm, coated with a layer of silica gel G. Prepare as follows: Weigh 30 g of silica gel G in a 300 ml conical flask, equipped with ground-glass stopper. Add 60 ml of distilled water, stopper the flask and homogenize the contents for 1 minute by shaking. Spread the suspension by means of a spreader onto 5 plates in such a way as to obtain a layer of 0.25 mm thickness (in the wet state). Allow the plates to dry in air for one-half hour and keep them in a drying-chamber at 60°C at least overnight until they are to be used.

20. Microcapillary pipettes of 2 micro-litres, Desaga or equivalent.

21. Spray bottle.

22. Oven, set at 100°C.

REAGENTS

1. Sea sand, washed in hydrochloric acid and calcinated.

2. Ethanol, 96%.

3. Petroleum ether, boiling range 40-60°C.

4. Basic aluminium oxide (Woelm or equivalent), activated for 1 hour at 400°C immediately before use.

5. Benzene (or substitute solvent).

6. Acetone.

7. Mixture of petroleum ether and acetone, 98:2 by volume. Measure exactly by pipetting 2 ml of petroleum ether from a filled 100 ml flask and replacing it with 2 ml of acetone.

8. Mixture of petroleum ether and acetone, 1:1 by volume. Measure 25 ml of petroleum ether and 25 ml of acetone, by graduated cylinder and mix.

9. Mixture of acetone and ethanol, 4:1 by volume. Measure 40 ml of acetone and 10 ml of ethanol by graduated cylinder and mix.

10. Ammonia (25% m/m NH_3).

11. Mixture of ethanol and ammonia, 2:1 by volume. Measure 40 ml of ethanol and 20 ml of ammonia 0.910, using a graduated cylinder and mix.

12. Solution of ethanolic potassium hydroxide 0.5N. Weigh 14 g of potassium hydroxide, dissolve in 500 ml of ethanol. Keep in the dark.

13. Diethyl ether, free from peroxides.

14. Anhydrous magnesium sulphate.

15. Sulphuric acid 8N.

16. Solutions of the reference colours, 0.5% in ethanol or chloroform. Dissolve 50 mg of each reference colour in 10 ml of ethanol, except carotene which must be dissolved in chloroform.

17. Mixture of n-hexane and ethyl acetate, 9:1 by volume.

18. Carr-Price Reagent. Weigh 25 g of antimony trichloride in a conical flask with a ground glass stopper and dissolve in 75 ml chloroform, taking care to exclude moisture.

PROCEDURE

Weigh 5 to 10 g of the sample in an aluminium dish containing sand, add 5 to 10 ml ethanol, stir, then leave the mixture in the oven overnight. Transfer the contents of the dish to a thimble or filter paper and extract with petroleum ether for 4 hours in a Soxhlet. Remove from the apparatus and evaporate the petroleum ether over a water bath.

Dissolve 0.5 g of the residue or 0.5 g of the oil or fat to be examined in 10 ml of petroleum ether in a 50-ml beaker. Place a plug of cotton-wool in a chromatography tube and push this down to just above the tap. Fill the tube with a suspension of aluminium oxide in benzene so as to obtain a column 10 cm in height. Elute the benzene, taking care to see that the column does not become dry. Rinse the column with 50 ml of petroleum ether or until all the benzene has been removed. Discard the benzene and rinsings. Add the colour solution and regulate the speed of elution to about 1 ml per minute. Rinse the column with 100 ml of petroleum ether. Do not allow the column to become dry. Discard the eluate.

Elute carotenes with 50 ml of the mixture of petroleum ether/acetone. Collect the eluate in a 100 ml round-bottomed flask. Evaporate under partial vacuum, using a rotary evaporator or a current of nitrogen, with the flask over a water bath. Take up the residue in 1 ml of diethyl ether and proceed with identification as below. Do not allow the column to become dry.

Elute amino-aniline colours with 50 ml of the mixture of petroleum ether/acetone 1:1. Collect the eluate in a 100 ml round-bottomed flask. Evaporate under partial vacuum using a rotary evaporator or by a current of nitrogen, with the flask on a water bath. Take up the residue in 1 ml of diethyl ether and proceed with identification. Do not allow the column to run dry.

Elute hydroxy-aniline colours with 50 ml of the acetone/ethanol mixture. Collect the eluate in a 100 ml round-bottom flask. Evaporate to dryness under vacuum using a rotary evaporator or on a water-bath in a current of nitrogen. Take up the residue with 1 ml of diethyl ether and proceed with identification. Avoid drying the column.

Elute bixine and hydroxy-aniline colours which may still remain on the column with 50 ml of the mixture of ethanol/ammonia (2:1). Collect the eluate in a 100 ml round-bottomed flask. Evaporate under partial vacuum using a rotary evaporator or in a current of nitrogen, with the flask on a water bath. Take up the residue in 1 ml of diethyl ether and proceed with identification.

Note that a change of colour of the aluminium column to a red-violet shade after the ethanol/ammonia mixture has been added indicates the presence of curcumin in the sample.

The presence of residual oil or fat in any of the above fractions can hinder identification by thin-layer chromatography. In this case, it is recommended to saponify the lipids present as indicated below.

Add to each of the residues 50 ml of ethanolic potassium hydroxide solution and some fragments of pumice-stone. Boil for 45 minutes under reflux. Cool and transfer the solutions into separate funnels using 100 ml of water. Carefully extract the aqueous phase of each (if it does not contain bixine) once with 50 ml and twice with 25 ml of diethyl ether. Wash the ethereal extracts three times, using 25 ml of water each time.

Dry each ether extract with anhydrous magnesium sulphate. Evaporate under partial vacuum using a rotary evaporator or a current of nitrogen. Take up the residue in 1 ml of diethyl ether and proceed with identification.

Conduct identification by thin-layer chromatography as follows:

Place 4 microlitres (or if need be, a larger quantity) of each of the ether solutions from above using a microcapillary pipette, along an imaginary line 2.5 cm away from the edge of the plate. Space the spots at intervals of 2 cm. In the same way, place 2 microlitres of solutions of reference colours. Develop the plate with benzene in a tank saturated with vapours of this solvent. Allow to migrate over a distance of 17 cm. The separation of the different colours may be facilitated by leaving the plate to dry in air after development, and by developing again with benzene. In certain cases, it is useful to repeat this procedure. (To separate Sudan I from Sudan II, develop with the mixture of n-hexane/ethyl acetate.)

Examine the plate and identify the colours, comparing the R_f values of the spots from the extracts with those of the reference solutions. After examination, place the plate in a tank containing enough Carr-Price reagent to saturate the tank with its vapour until the plate becomes visibly wet. A blue stain appearing in the suspected bixin fraction indicates the presence of bixin. Heat the plate for ten minutes to 100°C. The blue stain turns red-brown.

INTERPRETATION

Bixin is transformed by saponification into norbixin. If the suspected bixin fraction has been saponified, account must be taken of this during chromatographic examination, so that when comparison is made of the R_f values of the spots with those of the controls, it is useful to prepare a reference solution of norbixin.

As the carotenoids are likely to oxidise, it is necessary to spot the fractions containing them on to the chromatographic plate as quickly as possible, preferably under a current of carbon dioxide or nitrogen.

Oil Yellow GG and Oil Yellow XP may be encountered and can be distinguished as follows: The former is a mixture of a phenolic azo and bisazo dye, both components of which are extractable from ether by caustic alkali. This behaviour distinguishes it from Oil Yellow XP, a pyrazolone dye, and from many other oil-soluble colours. It also means that it cannot be separated from an oily mixture by saponification followed by extraction of the non-saponifiable fraction, which is a useful means of isolating many other colours of this type. However, if a solution of the fat in ether is extracted with aqueous sodium hydroxide before saponification this difficulty is largely overcome, as Oil Yellow GG will then be removed.

Oranges may be examined for Oil Orange XO by removing the peel and soaking it in a 50:50 mixture of acetone and ether, decanting the coloured solvent and evaporating it nearly to dryness. The residue is then taken up with a mixture of water and ether and transferred to a separating funnel where it is shaken. After separation the aqueous layer is discarded. The ether is washed with a dilute solution of caustic soda and then with water until neutral. It is dried with anhydrous sodium sulphate and then evaporated. The residue is taken up in petroleum ether (boiling range 40°-60°C) and is run through a 0.5 cm by 4 cm column of BDH chromatographic alumina or equivalent grade. The column is well washed with petroleum ether and then with petroleum ether containing 5 percent of diethyl ether, which causes the dye to travel down the column as a red band. This coloured fraction is collected, the solvent is removed and the residue is dissolved in chloroform for examination in a spectrophotometer. In this way correspondence with the standard curve can be obtained right into the ultra-violet.

REFERENCE

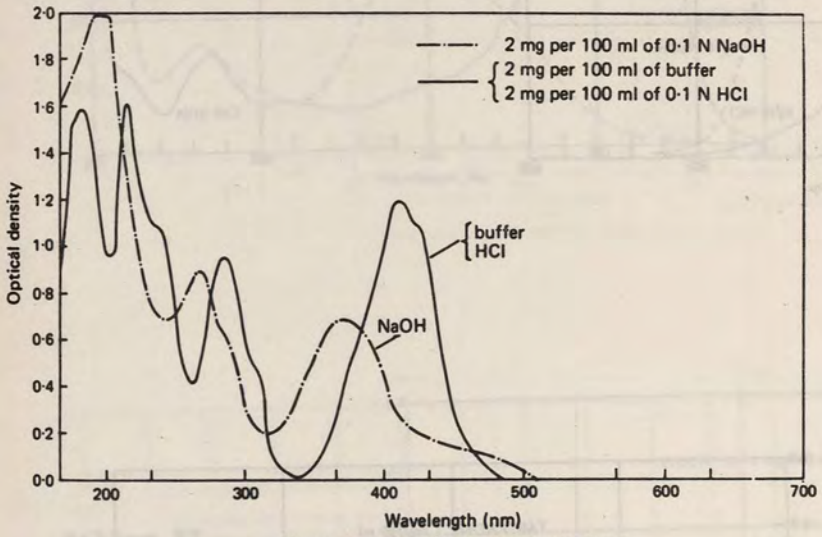
EEC Document 1867/VI/72/F/Rev.1.

COLOUR IDENTIFICATION BY SPECTRA

If, after separation and attempted identification by chromatography, the colour appears to be a non-permitted one, it must be identified before an adverse report can be given. The first step is to cut out the paper spot or scrape the spot off the TLC plate and dissolve the dye in a suitable solvent, water or acetone-alcohol. A useful device for the removal of spots from TLC plates is described by Sahasrabudhe in JAOAC 47 1964 (889). After centrifuging or filtration, remove organic solvents by gentle evaporation and dissolve in 0.1 N NaOH or adjust the strength of an aqueous solution to 0.1 N with alkali and read the spectrum from about 210 nm to 650 nm. This solution, or a fresh one, should be adjusted to 0.1 N with hydrochloric acid and the readings repeated. The plotted curves may be compared with those reproduced below.

The following spectra were taken from "Separation and Identification of Food Colours Permitted by the Colouring Matter in Food Regulations 1957", by the Association of Public Analysts (U.K.), 1960.

Included with each spectra are the usual name, the Colour Index Number, occasionally the EEC number (in parenthesis), and synonyms and other names (also in parentheses).



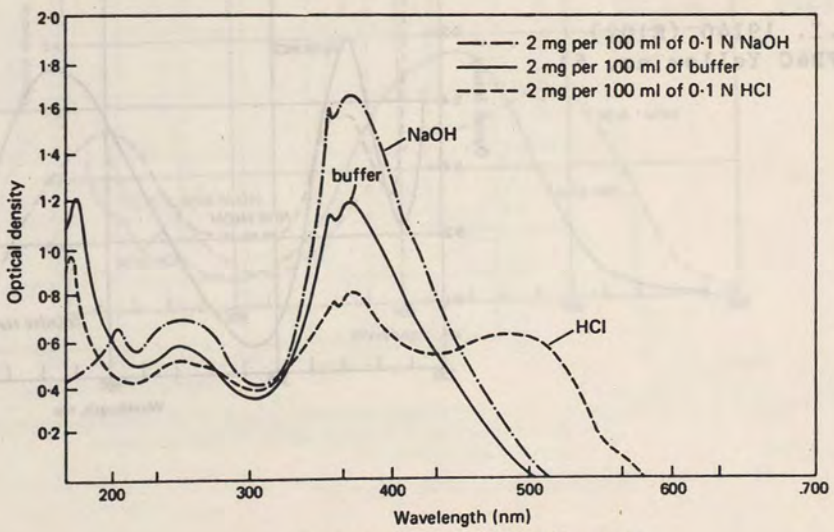
Absorption curve for Quinoline Yellow.

Quinoline Yellow

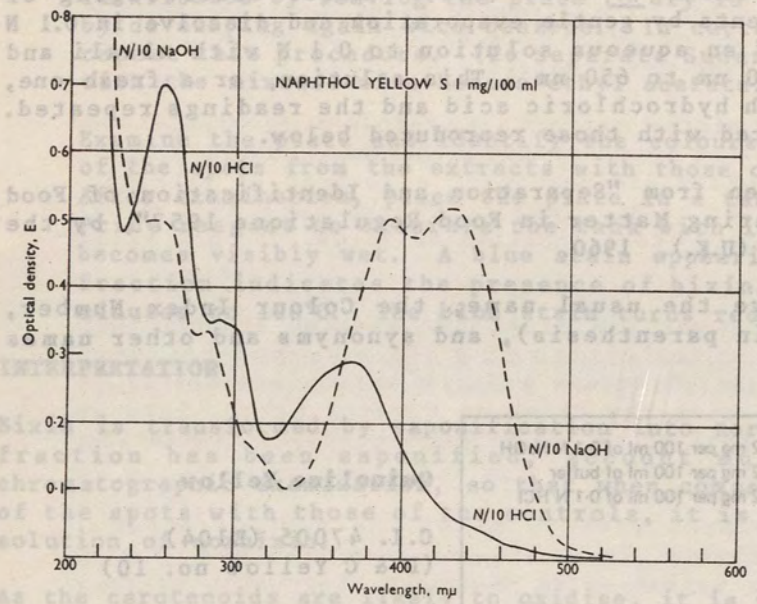
C.I. 47005 (E104)
(D & C Yellow no. 10)

Fast Yellow AB

C.I. 13015
(Acid Yellow G)

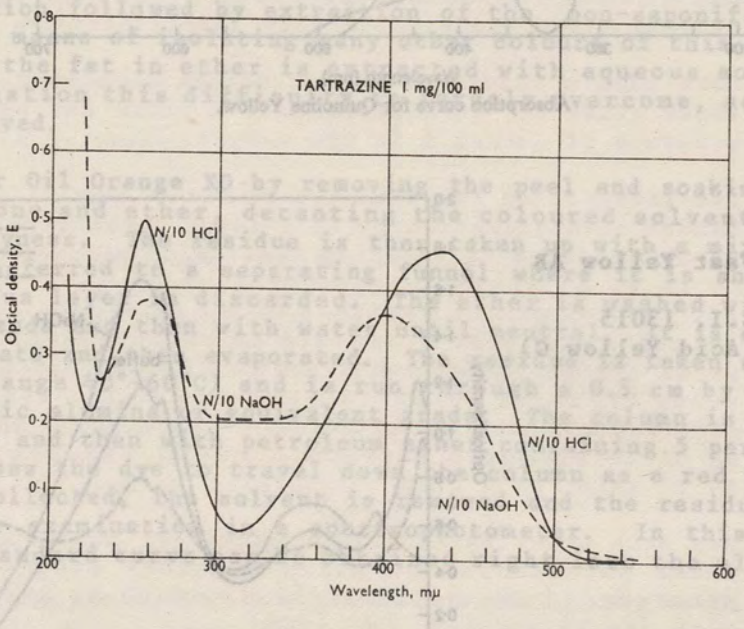


Absorption curve for Fast Yellow AB.



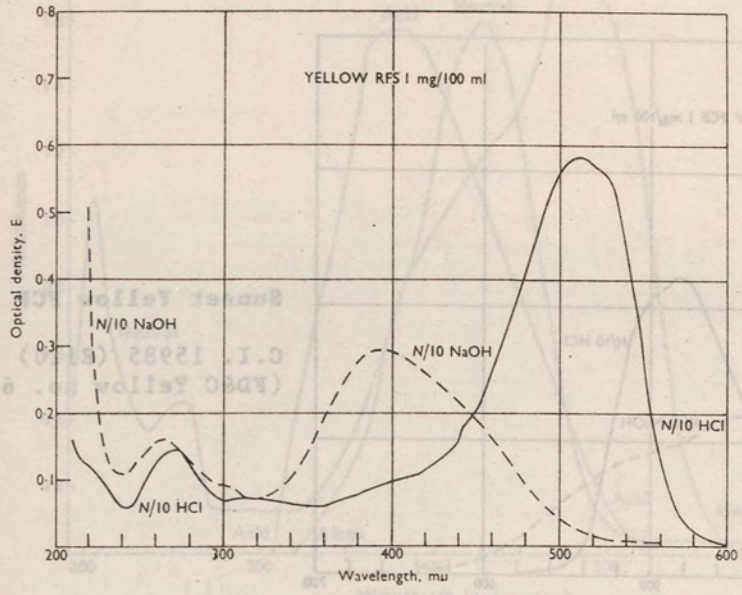
Napthol Yellow

C.I. 10316
(FD&C Yellow no. 1 & 2)



Tartrazine

C.I. 19140 (E102)
(FD&C Yellow no. 5)

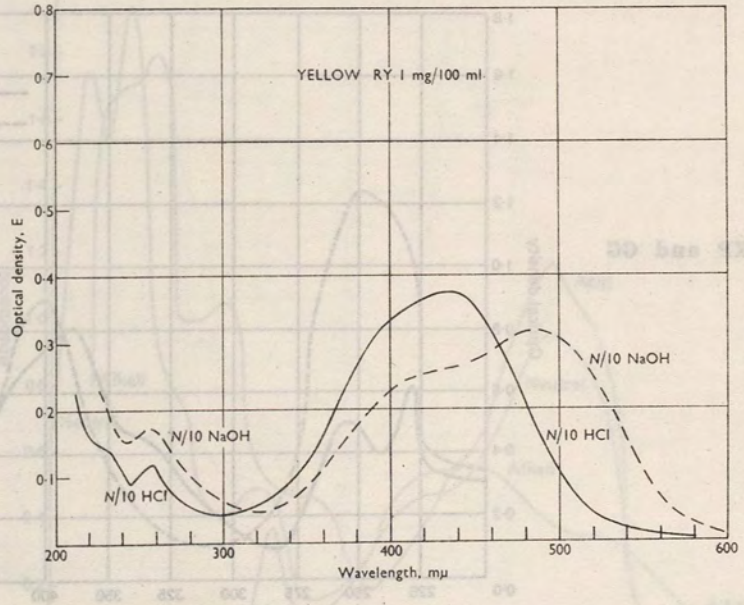


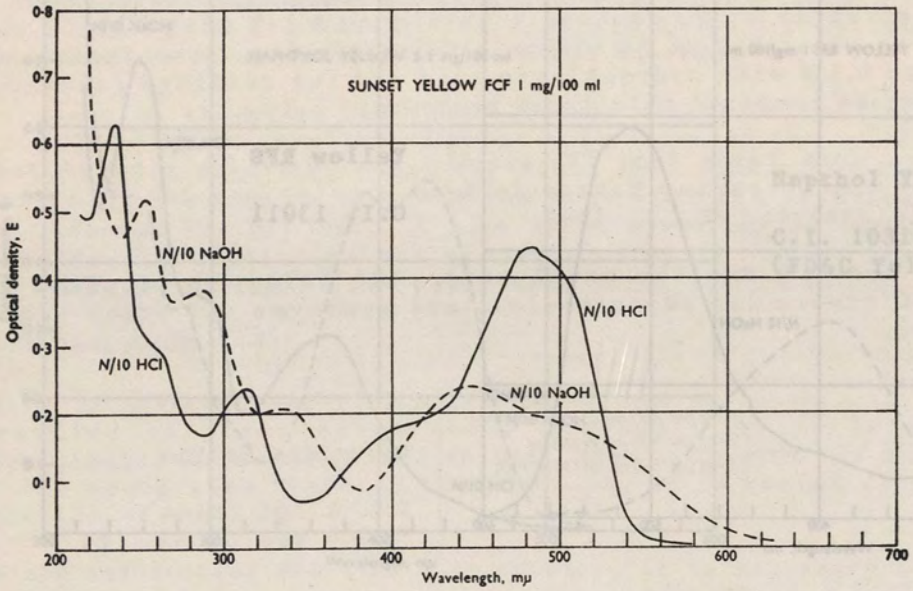
Yellow RFS

C.I. 13011

Yellow RY

C.I. 14330



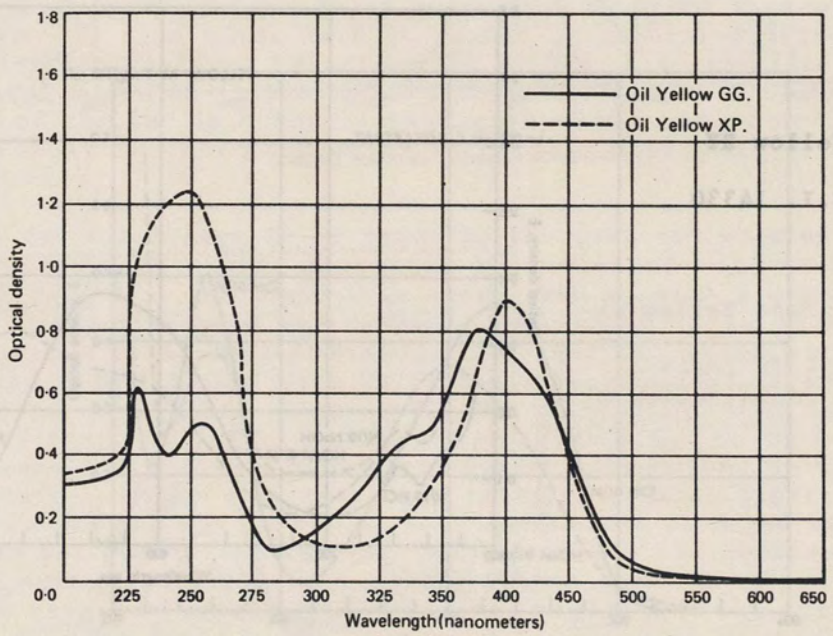


Sunset Yellow FCF

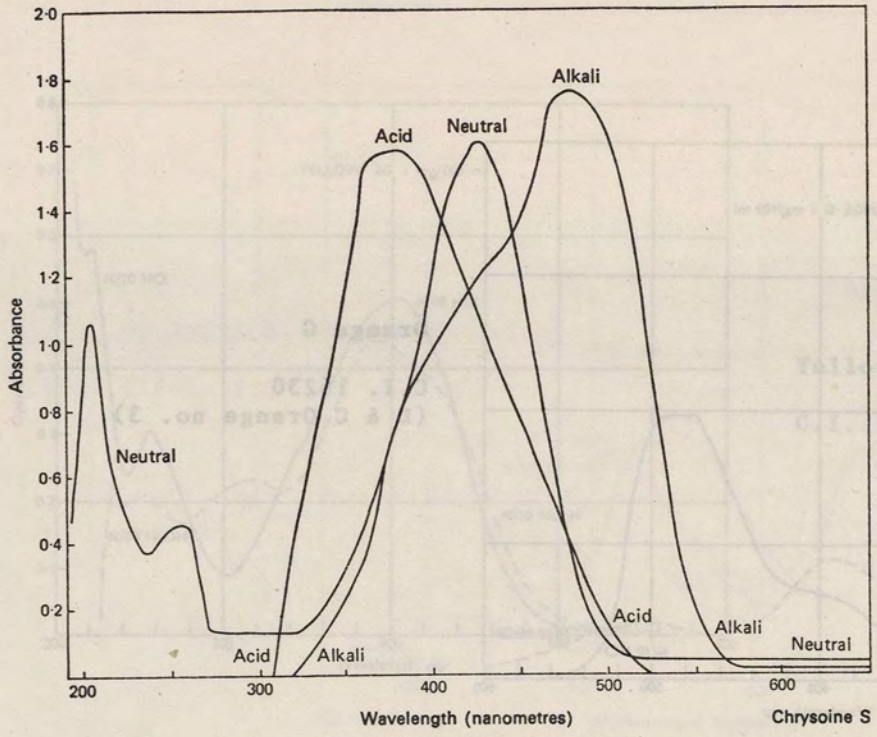
C.I. 15985 (E110)
(FD&C Yellow no. 6)

Oil Yellow XP and GG

C.I. 12740



Absorption curve for permitted oil-soluble colours Oil Yellow GG and Oil Yellow XP at concentrations of 2 mg per 100 ml of light petroleum.



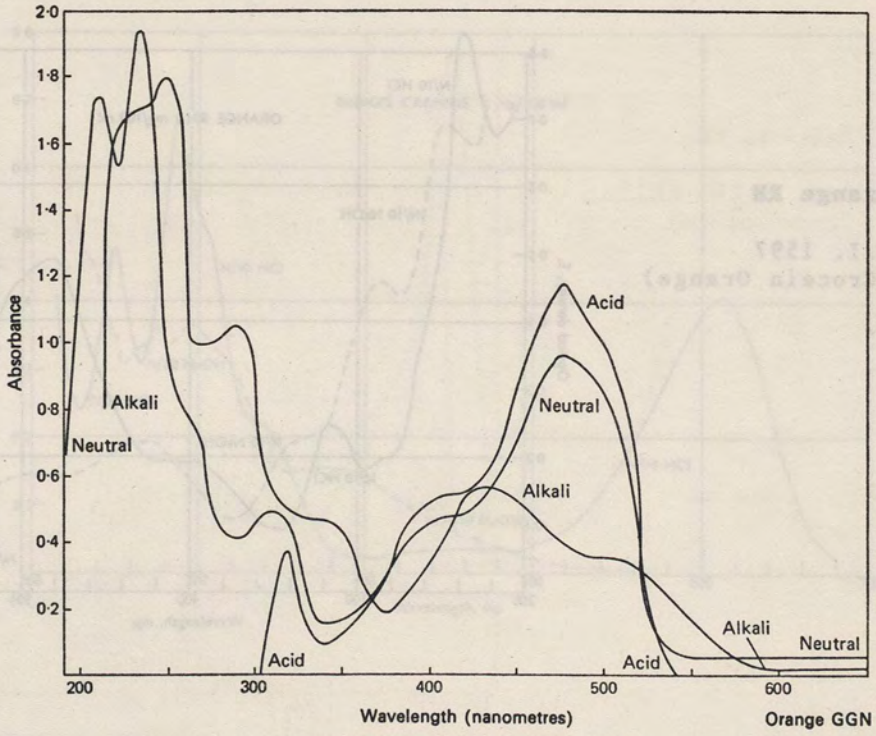
Absorption curves for Chrysoine S

Chrysoin S

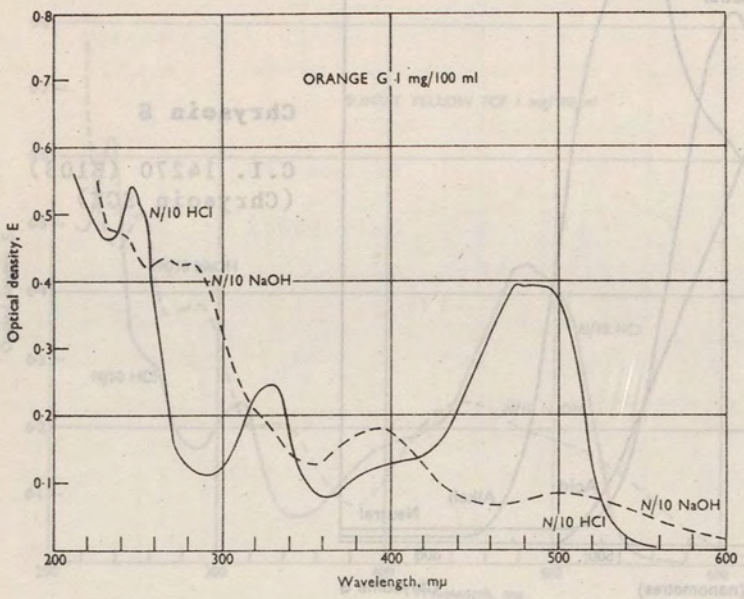
C.I. 14270 (E103)
(Chrysoin SGX)

Orange GGN

C.I. 15980 (E111)
(Orange GGL or GGP)

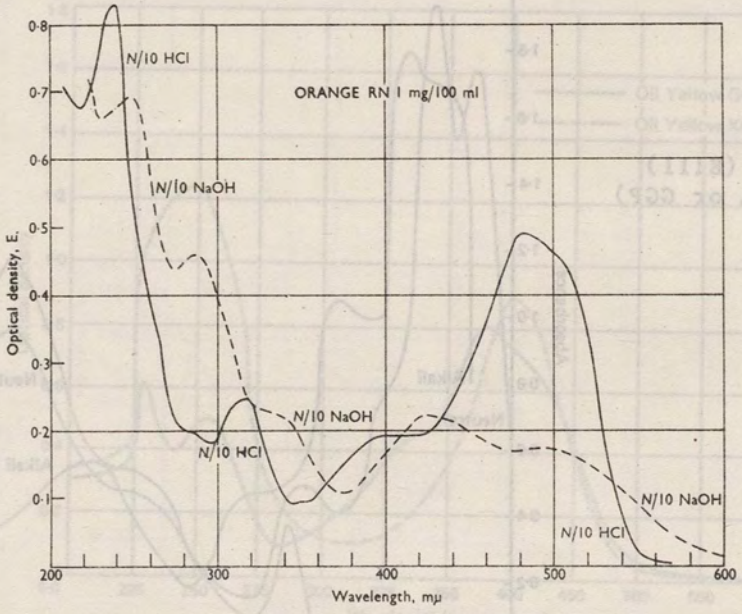


Absorption curves for Orange GGN



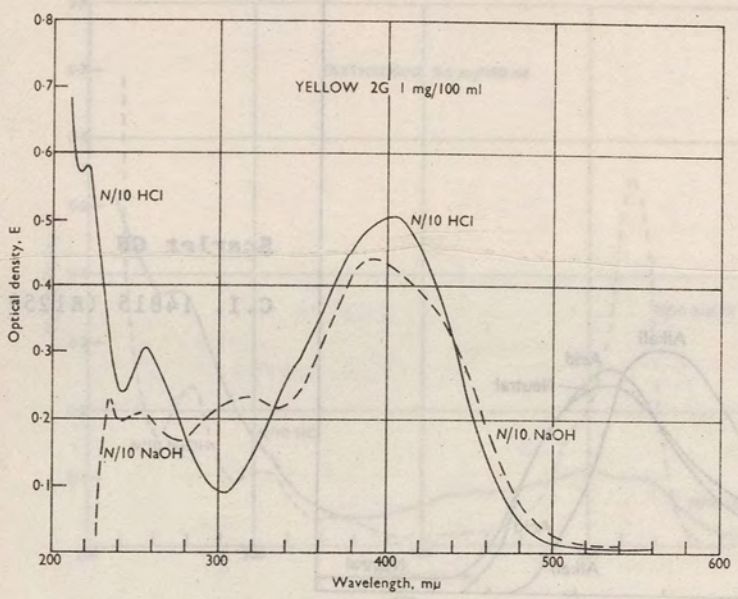
Orange G

C.I. 16230
(D & C Orange no. 3)



Orange RN

C.I. 1597
(Crocein Orange)

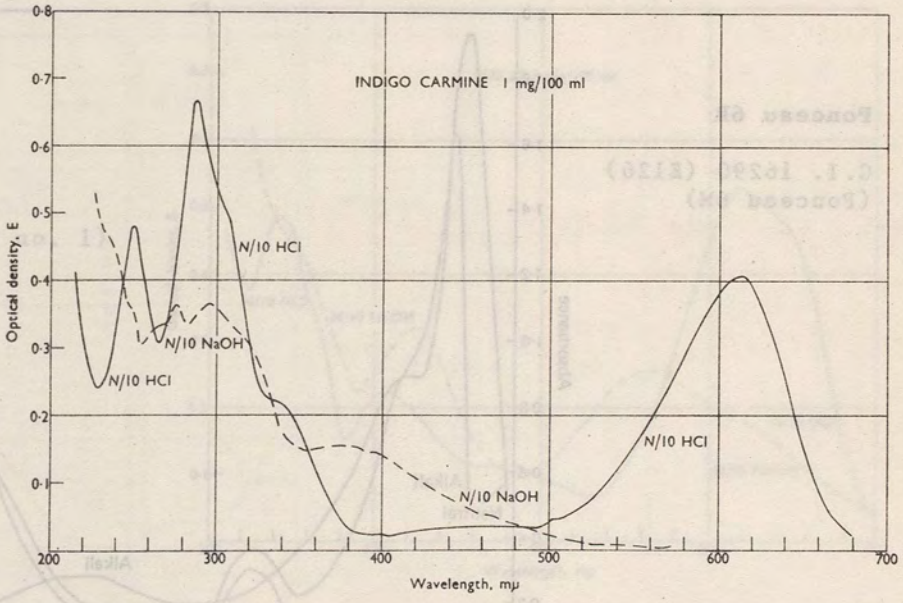


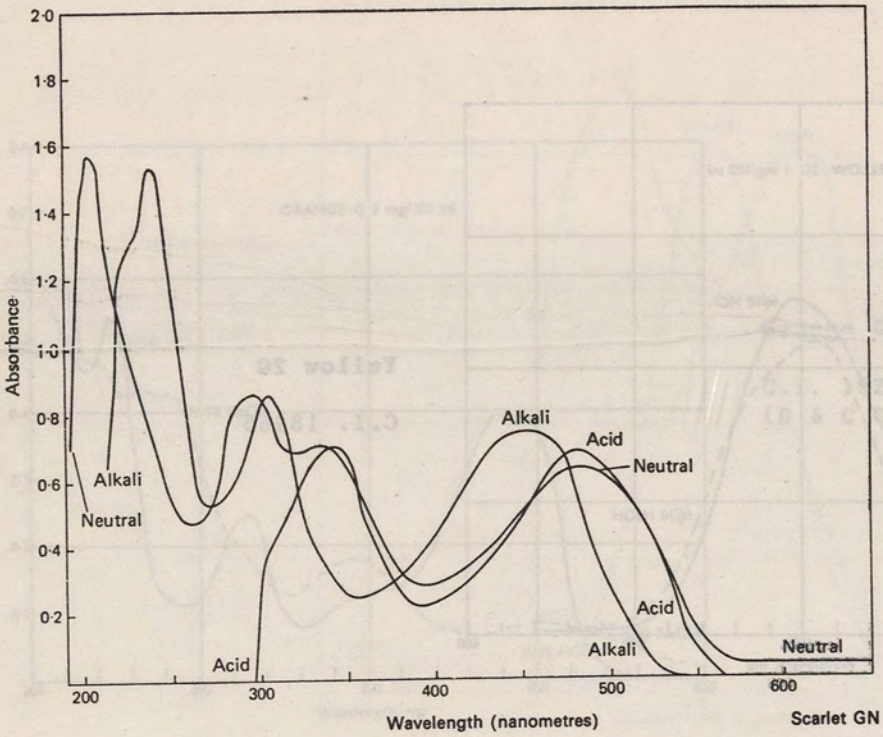
Yellow 2G

C.I. 18965

Indigo Carmine

C.I. 73015 (E132)
(FD&C Blue no. 2)
(Indigoline)





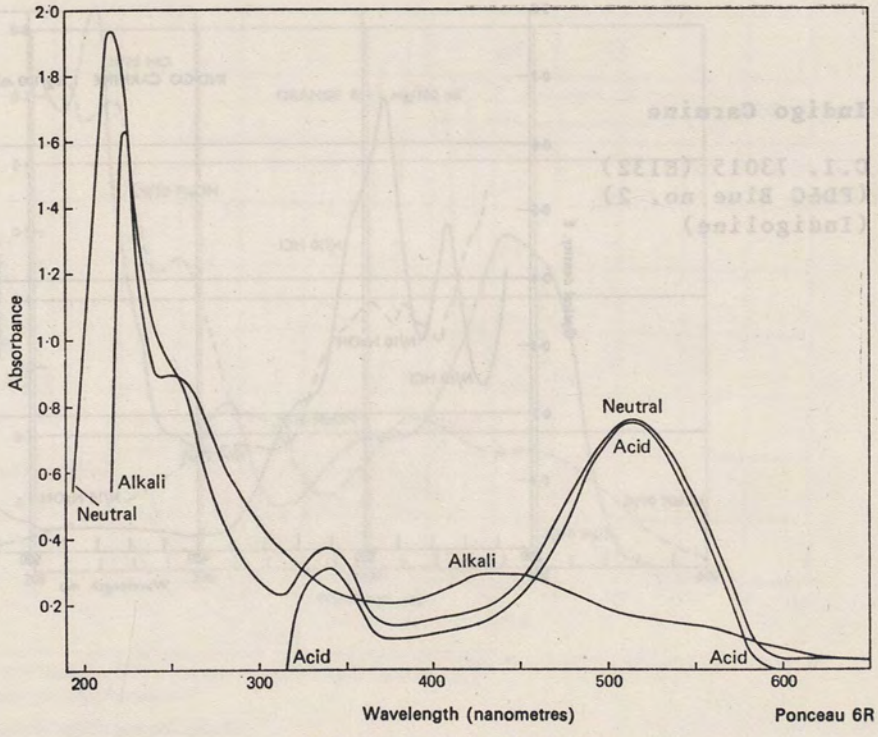
Absorption curves for Scarlet GN

Scarlet GN

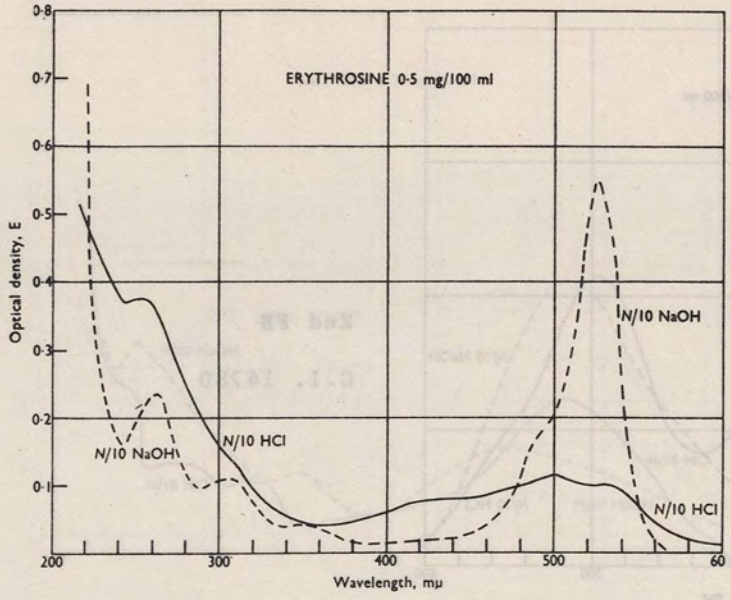
C.I. 14815 (E125)

Ponceau 6R

C.I. 16290 (E126)
(Ponceau 6M)



Absorption curves for Ponceau 6R

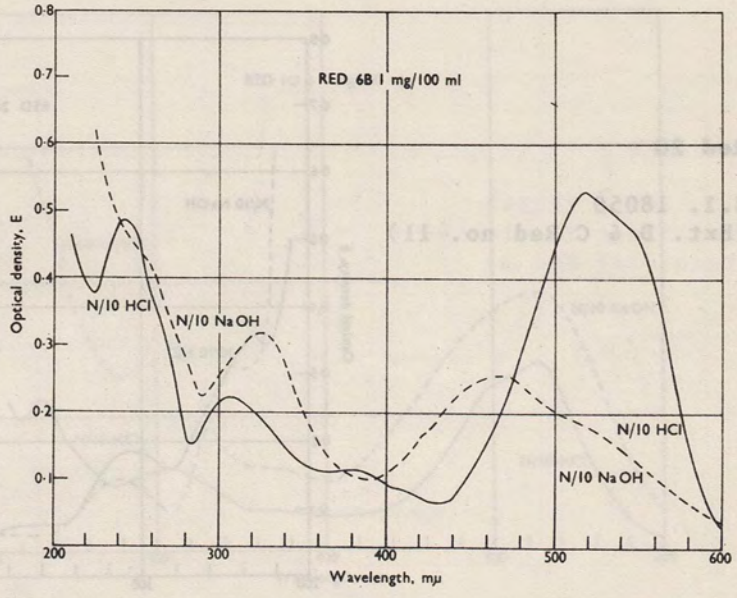


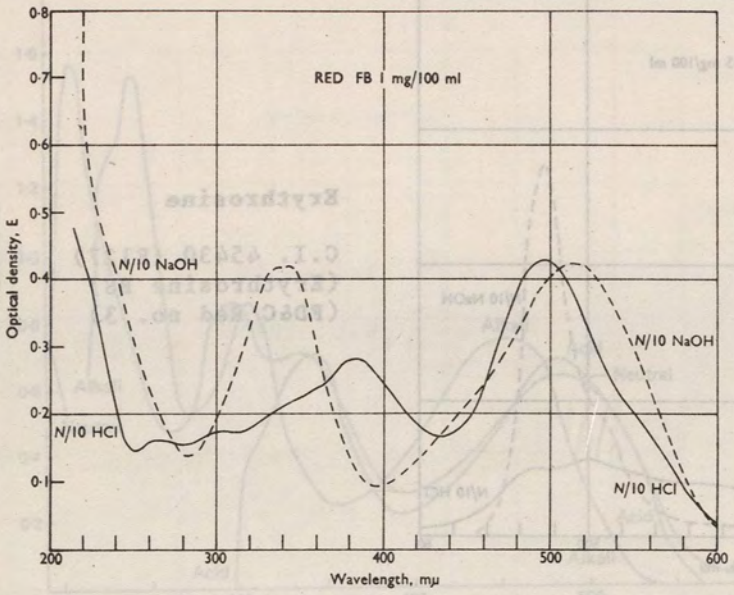
Erythrosine

C.I. 45430 (E127)
(Erythrosine BS)
(FD&C Red no. 3)

Red 6B

C.I. 18055
(Ext. D & C Red no. 1)



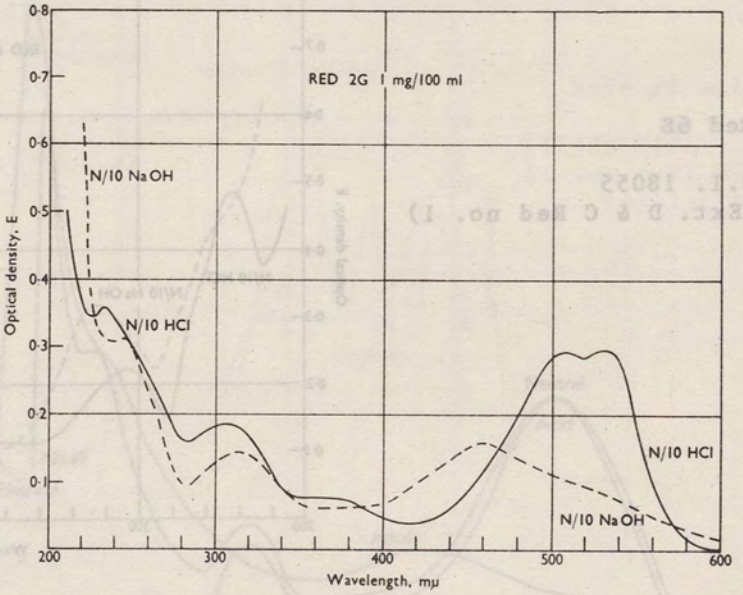


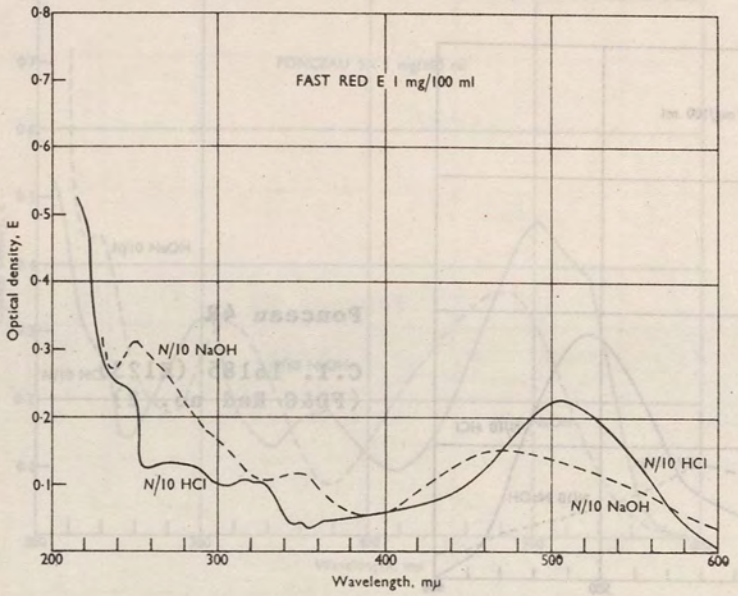
Red FB

C.I. 14780

Red 2G

C.I. 18050
(Ext. D & C Red no. 11)



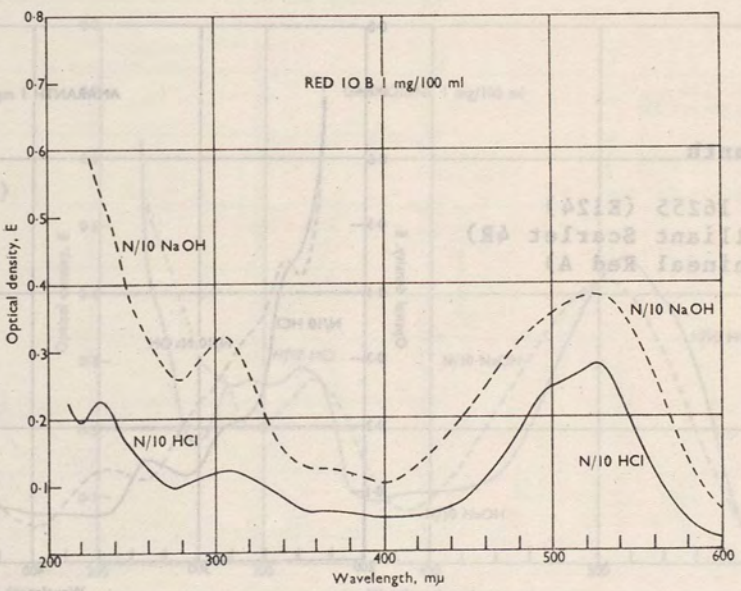


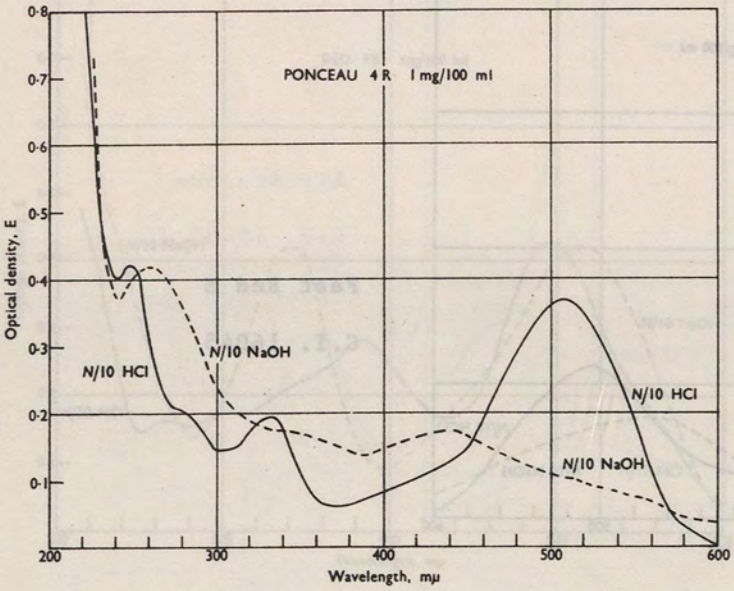
Fast Red E

C.I. 16045

Red 10B

C.I. 17200
(D & C Red no. 33)



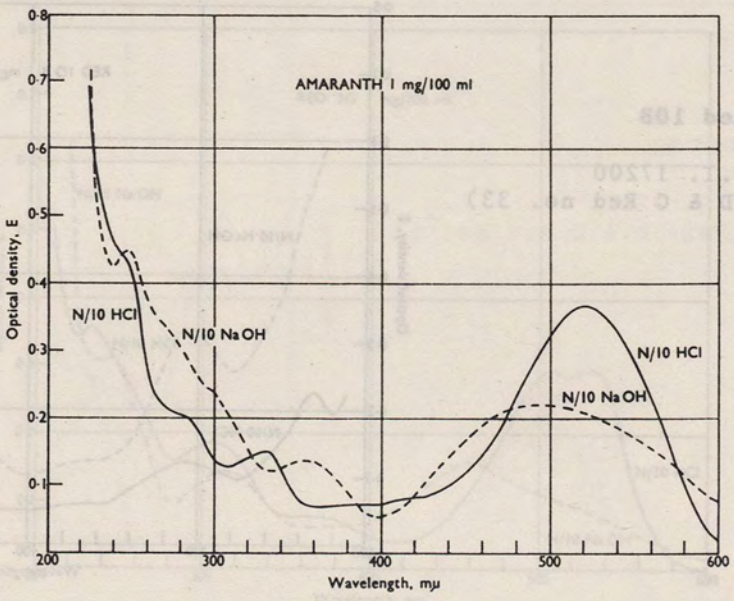


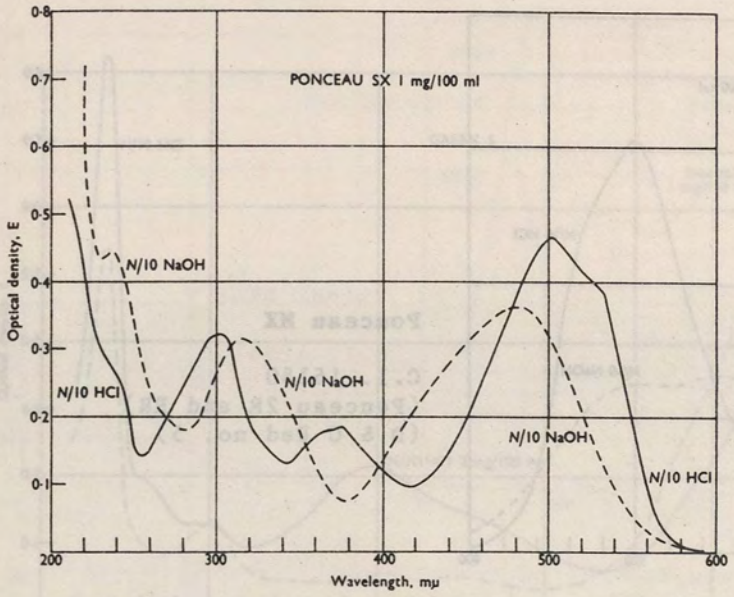
Ponceau 4R

C.I. 16185 (E123)
(FD&C Red no. 2)

Amaranth

C.I. 16255 (E124)
(Brilliant Scarlet 4R)
(Cochineal Red A)



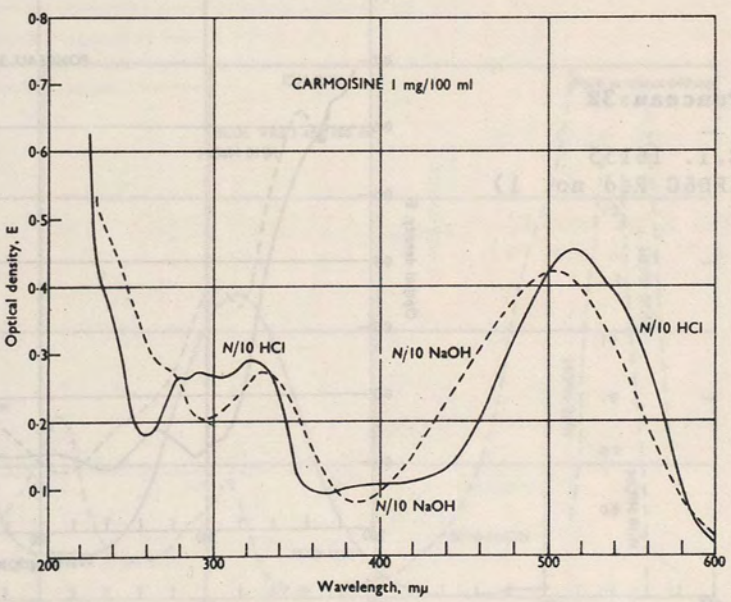


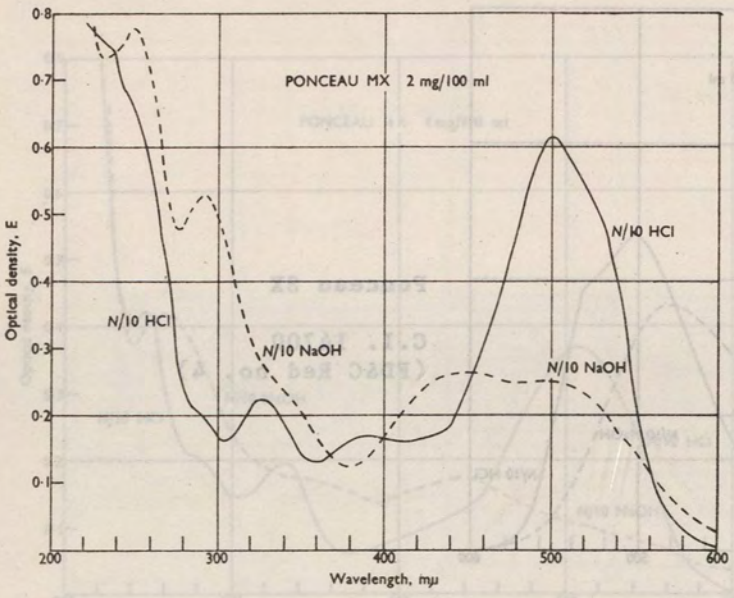
Ponceau SX

C.I. 14700
(FD&C Red no. 4)

Carmoisine

C.I. 14720 (E122)
(Ext. D & C Red no.10)



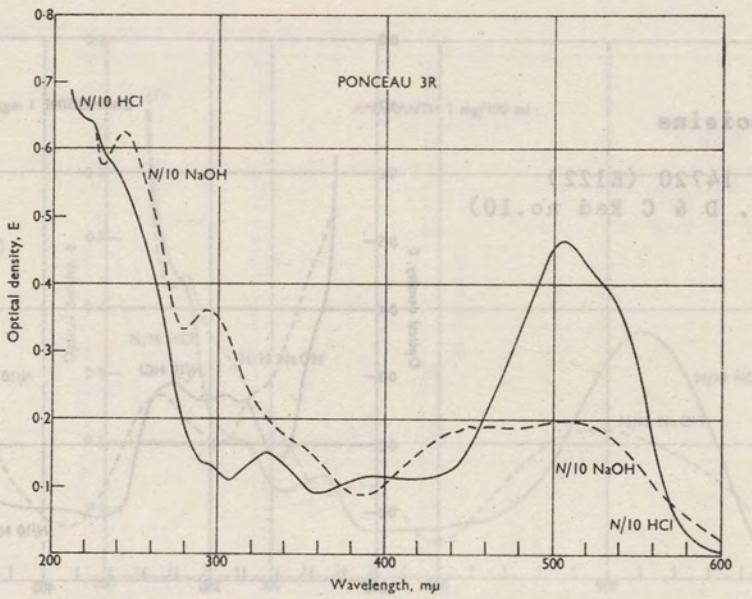


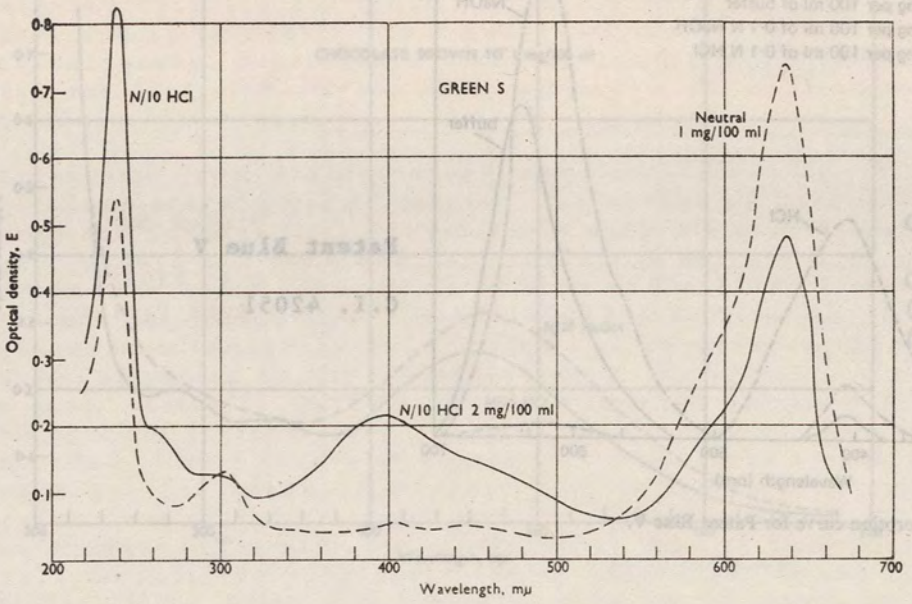
Ponceau MX

C.I. 16150
(Ponceau 2R and RR)
(D & C Red no. 5)

Ponceau 3R

C.I. 16155
(FD&C Red no. 1)



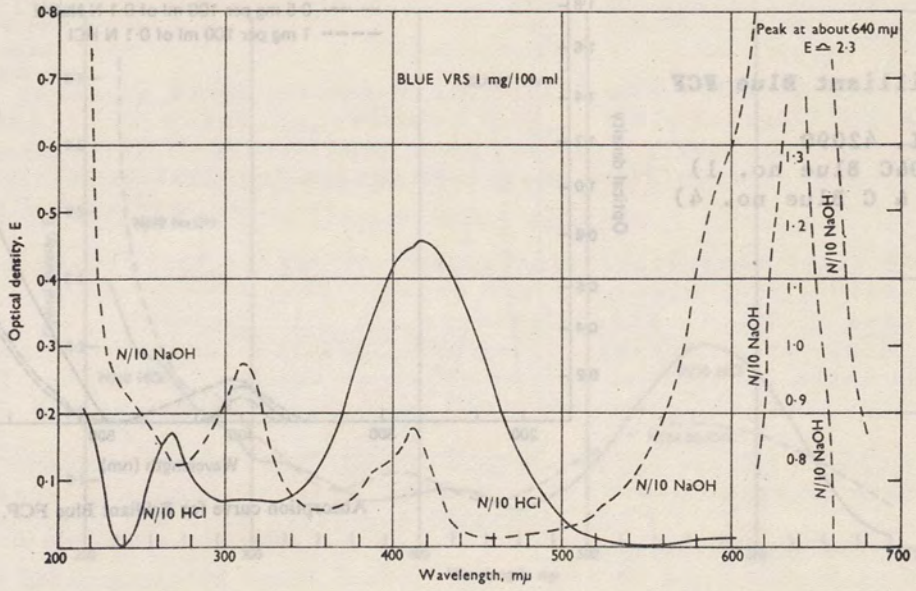


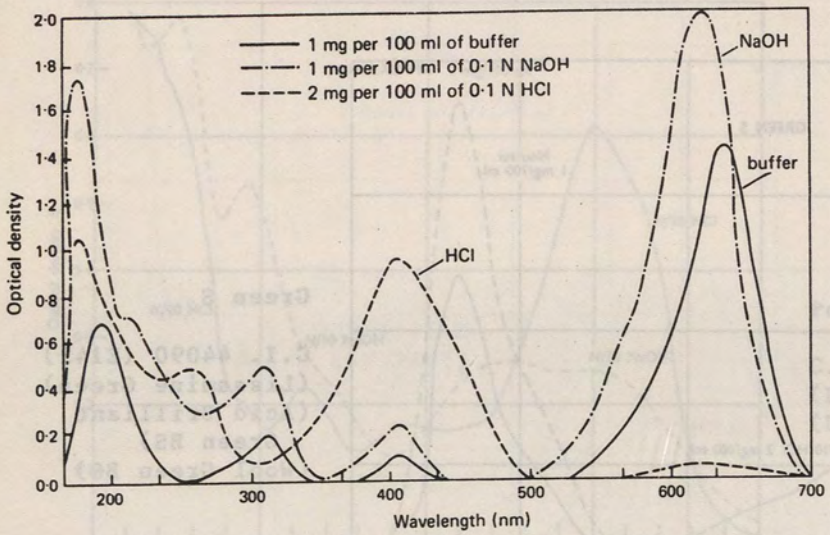
Green S

C.I. 44090 (E142)
(Lissamine Green)
(Acid Brilliant
Green BS)
(Wool Green BS)

Blue VRS

C.I. 42045





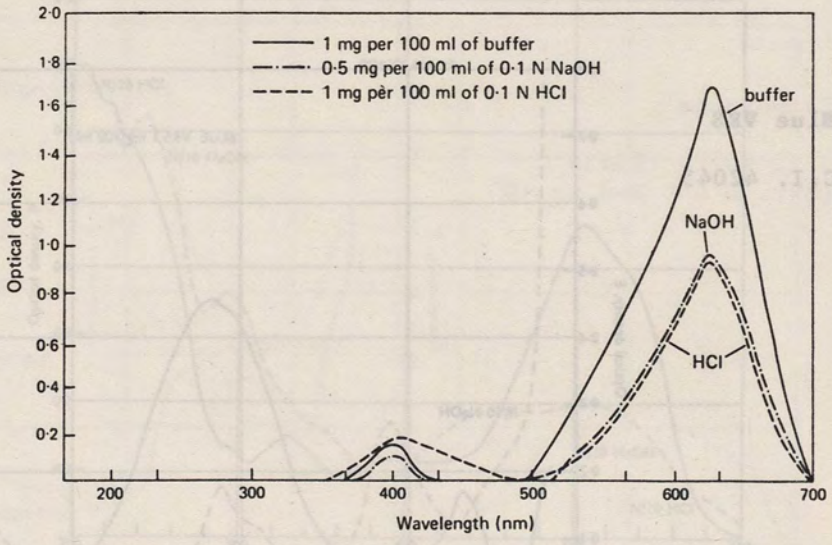
Absorption curve for Patent Blue V.

Patent Blue V

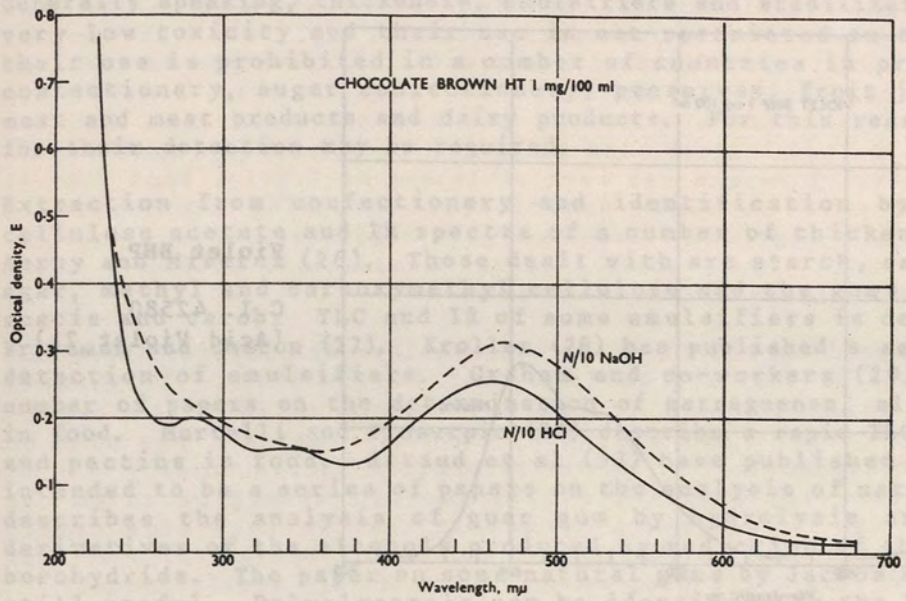
C.I. 42051

Brilliant Blue FCF

C.I. 42090
(FD&C Blue no. 1)
(D & C Blue no. 4)



Absorption curve for Brilliant Blue FCF.

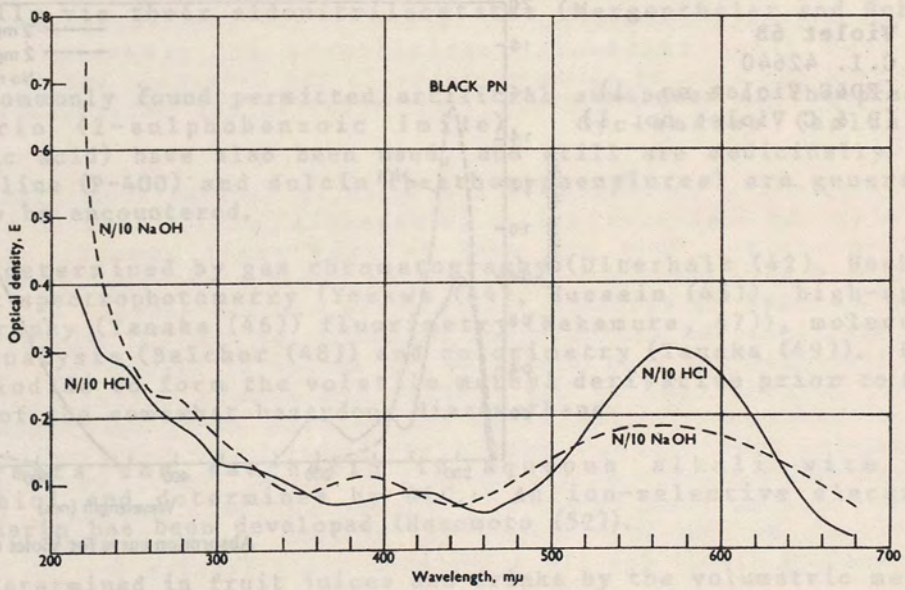


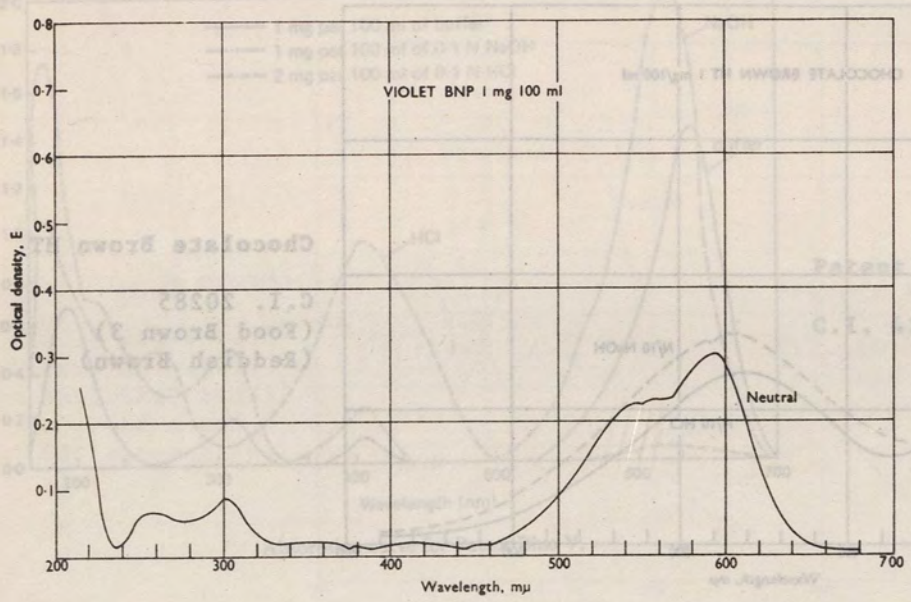
Chocolate Brown HT

C.I. 20285
(Food Brown 3)
(Reddish Brown)

Black PN

C.I. 28440 (E151)
(Food Black 1)

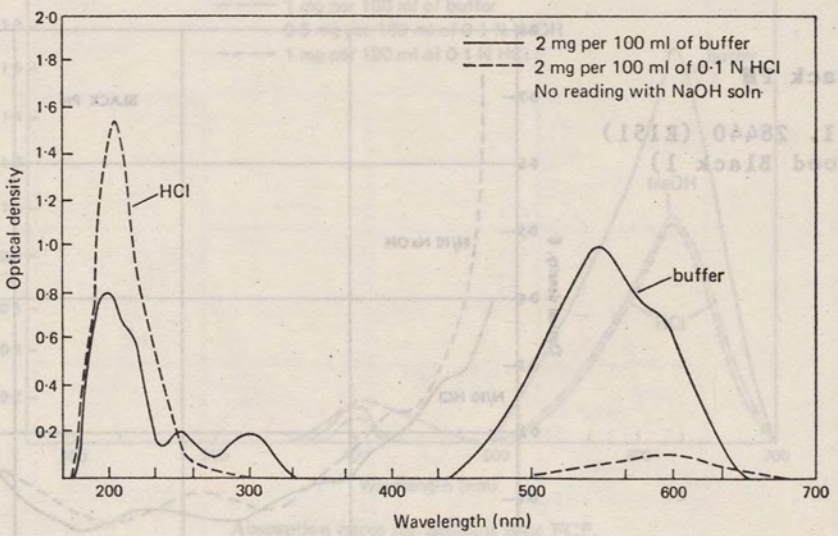




Violet BNP

C.I. 42580
(Acid Violet 21)

Violet 6B
C.I. 42640
(FD&C Violet no. 1)
(D & C Violet no. 1)



Absorption curve for Violet 6B.

6.4 OTHER ADDITIVES

Preservatives, antioxidants and colours are the most common food additives. However, there are many other additives which are incorporated into food to improve appearance, taste and physical character.

Generally speaking, thickeners, emulsifiers and stabilizers used in food are of very low toxicity and their use is not restricted on this basis. However, their use is prohibited in a number of countries in products such as flour confectionery, sugar confectionery, preserves, fruit juices and soft drinks, meat and meat products and dairy products. For this reason, qualitative tests for their detection may be required.

Extraction from confectionery and identification by electrophoresis on cellulose acetate and IR spectra of a number of thickeners are described by Aerny and Miserez (26). Those dealt with are starch, carrageenan, alginate, agar, methyl and carboxymethyl cellulose and the gums of guar, tragacanth, acacia and carob. TLC and IR of some emulsifiers is described by Srebrnik-Friszman and Charon (27). Kroller (28) has published a series of papers on the detection of emulsifiers. Graham and co-workers (29)(30)(31) published a number of papers on the determination of carrageenan, alginate and other gums in food. Martelli and Proserpio (32) describe a rapid TLC method for alginates and pectins in food. Artaud et al (33) have published the first of what is intended to be a series of papers on the analysis of natural gums. The paper describes the analysis of guar gum by hydrolysis and GLC of the silyl derivatives of the alcohols produced by reduction of the sugars with sodium borohydride. The paper on some natural gums by Jacobs and Jaffe (34) is also still useful. Polyglycerols can be identified by the TLC of their acetates (Dallas (35)). Seher (36) published a series of papers on the analysis of non-ionic surface active agents dealing with the IR spectra of partial glycerides. Gernert (37) used TLC to detect the glycerides of lactic, tartaric and citric acids in food. Stearoyl lactates may be detected by the TLC method of Regula (38). For determination of polysorbate 60 in a number of foods, using a gravimetric finish with barium phosphomolybdate, see Smullin et al (39). Murphy and Scott (40) describe the determination of polyoxyethylene emulsifiers by TLC after column clean-up. Monosaccharides obtained by hydrolytic cleavage of polysaccharide-containing stabilizers have been separated gas chromatographically via their aldonitrilacetates (Mergenthaler and Scherz (41)).

By far the most commonly found permitted artificial sweetener at the present time is saccharin (2-sulphobenzoic imide). Cyclamates (salts of cyclohexylsulphamic acid) have also been used, and still are medicinally. 5-Nitro-1-propoxyaniline (P-400) and dulcin (p-ethoxyphenylurea) are generally prohibited but may be encountered.

Saccharin can be determined by gas chromatography (Ulterhalt (42), Hashiro (43)), ultra-violet spectrophotometry (Yozawa (44), Hussein (45)), high-speed liquid chromatography (Tanaka (46)) fluorimetry (Nakamura, 47)), molecular emission cavity analysis (Belcher (48)) and colorimetry (Tanaka (49)). Doun (50) uses methyl iodide to form the volatile methyl derivative prior to GLC, thus avoiding use of the somewhat hazardous diazomethane.

Makita (51) treats the saccharin in aqueous alkali with the trichloromethylthiol and determines by GLC. An ion-selective electrode sensitive to saccharin has been developed (Hazemoto (52)).

Cyclamate may be determined in fruit juices and drinks by the volumetric method of Davies (53). Meadows (54) describes a volumetric method for cyclamates in sweetening tablets. Harrison and Cook (55) describe a colorimetric method suitable for both types of sample. Colorimetric methods are also described by Dawana (56) and Matsui (57) and GLC methods by Groebel and Wessels (58) and Mori (59).

Takeshita (60)(61) uses column and TLC to detect saccharin, cyclamate and dulcin in a variety of foods. Nagasawa et al (62) use TLC alone. Das et al (63)(64) describe TLC and PC methods. Sasaki et al (65) describe the detection of dulcin by PC.

A number of polyols (naturally occurring polyhydric alcohols) are used in food, especially that intended for diabetics. The most important members of this class are sorbitol and mannitol and, more recently, xylitol. If used to replace sugar they will be present in quite substantial proportions as they are less sweet. They are also used as humectants and sequestrants. Some simple peptides and their derivatives are sweet, for example Aspartame (sodium aspartyl phenylalanine methyl ester), and Suosan, N-(p-nitrophenyl carbamoyl)-beta-alanine sodium salt. A review has been published by British Food Journal 77 5-6 (1975) on a number of sugar substitutes including neohesperidin dihydrochalcone and some dipeptides. Ammonium glycyrrhizinate, from liqurice, is another sweetening agent, more usually found in medicinal products. This may be determined by GLC (Larry (66)).

Sorbitol may be determined by GLC of the trimethylsilyl derivatives (Blum and Koehler (67), Jones, et al (68) and Williams and Martin (69)). Polyhydric alcohols react with borax to form relatively strong acids which can be titrated. This is the basis of the classical method. Sorbitol may also be reacted with sodium periodate and the excess determined iodometrically (Van Os and Elena (70)). Graham (71) suggests a colorimetric method based on the Komarowsky reaction, the colour being formed in the presence of a cyclic aldehyde, thiourea, concentrated sulphuric acid and heat. Konig (72) describes a GLC method for determining cyclamate, saccharin and dulcin simultaneously.

A very large number of additives are now accepted for use in foods for a wide variety of purposes. The additives may change, react with the food or disappear during processing and storage so that analysis may be difficult or impossible. In many cases the chemical in question is already present in the food but more is added during processing. Enforcement of food law does not necessarily require the detection or determination of additives, such as those known to be present in amounts accepted as innocuous or for which no limit is prescribed. Some additives can be determined or the amount present deduced from general methods of analysis such as acidity or ash. The Food Additives and Contaminants Commission of IUPAC (73) reviewed analytical methods available for some flour improvers, antioxidants and preservatives. Walker (74) discusses the role of chromatography in the detection and determination of food additives. Clark and Robinson (75) describe the determination of surfactants in food.

Most flavouring materials were originally derived from plants in the form of extracts and distillates such as essential oils. Some of these are now made synthetically. They are used in only small amounts and were generally considered safe until recently, but now doubts are expressed about a few. For example, there is evidence that safrole and iso-safrole are carcinogenic. The Food Additives and Contaminants Committee of the U.K. Ministry of Agriculture, 1976 Fisheries and Food Report on the Review of Flavourings in Food serves as a useful introduction to legislative and manufacturing considerations. The matter has been considered by the Working Party on Natural and Artificial Flavouring Substances, a subsidiary body of the Sub-Committee on the Health Control of Foodstuffs of the Council of Europe (EEC). There was a symposium on flavourings in food in 1976, reported in "International Flavours and Food Additives", Nov/Dec 1976 issue. The FAO/WHO Expert Committee on Food Additives 11th Meeting (1967) details acceptable daily intakes for 35 flavours.

Monosodium glutamate belongs to a group of compounds that function as flavour enhancers. Ingestion of quite large amounts results in a temporary headache and "hot flushes". Fernandez-Flores, Johnson and Blomquist (76)(77) reported on a method studied collaboratively, using separation on an ion-exchange column followed by a Sorensen formaldehyde titration. Baily and Swift (78) describe a

paper chromatography method, and Gal and Schilling (79) one using GLC. Coppola, Christie and Hanna (80) describe a rapid method using a short ion-exchange column with the determination conducted fluorimetrically.

EMULSIFIERS
(Thin-layer Chromatography Method)

PRINCIPLE

Emulsifiers, together with fat, are extracted by blending with chloroform and methanol. On adding a calculated amount of water, the chloroform containing fat and emulsifier separates. The chloroform layer is evaporated to dryness and emulsifiers present extracted with methanol. The methanol extract is examined using thin-layer chromatography (TLC).

APPARATUS

1. TLC equipment, including Polygram Sil G. plates (manufactured by Macherey Nagel and Co., 5161 Buren, Wertstrasse 628, Federal Republic of Germany).

2. Short-wave U.V. lamp, 254 nm.

3. Rotary film evaporator.

REAGENTS

1. Chloroform.

2. Methanol.

3. Magnesium chloride solution, 2N.

4. 1,2-Dichloroethane.

5. Cyclohexane.

6. Butan-2-one.

7. Glacial acetic acid.

8. Dibromofluorescein, 0.02 percent in 96 % ethanol.

9. TLC Solvent 1: Pipette 12 ml MeOH into a dry 200 ml volumetric flask and dilute to the mark with 1,2-dichlorethane.

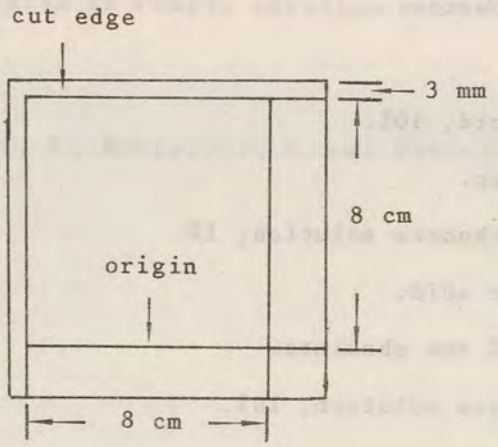
10. TLC Solvent 2: Pipette 4 ml water and 16 ml acetic acid into a dry 200 ml volumetric flask. Add from a measuring cyclinder 80 ml dry butan-2-one and dilute to the mark with cyclohexane (this solvent separates below about 15°C).

PROCEDURE

Into a dry macerator goblet, put about 20 g sample, 40 ml $CHCL_3$, 80 ml MeOH and 4.0 ml 2 N $MgCL_2$ solution. Macerate about 2 min. Add a further 40 ml $CHCl_3$ and macerate about 2 min more. Filter (Whatman No. 1 filter paper or equivalent). A reasonably clear filtrate should be obtained. Wash the goblet with $CHCl_3$ and pass through the filter. Transfer the filtrate to a 250 ml separator. Add sufficient water to make a total of 72 ml taking into account the moisture content of the sample. Mix thoroughly and allow to stand until the $CHCl_3$ has completely separated and is clear (takes at least one hour). Run off the $CHCl_3$ into a tared round-botomed flask. Evaporate on a water bath and dry at 100°C to obtain an estimate of the fat and emulsifier content. Add sufficient MeOH to give a 10 percent solution. Heat to boiling on a water bath. Stopper and shake the flask. Unstopper and heat again to boiling then allow to stand and cool for about 10 minutes until the undissolved fat has

settled. Decant off the methanol into a tared flask, evaporate on a water bath, then dry at 100°C to give the weight of emulsifier concentrate. Dissolve concentrate in $CHCl_3$/MeOH 1:1 to give a ten percent solution.

TLC plates supplied as 20 x 20 cm need washing to remove fluorescent impurities. This is done by developing them with $CHCl_3$/MeOH 1:1 in an unlined tank for about 24 hours. Dry at 100°C for about half an hour. The plate is then cut into quarters. The cut edges are tidied by removing a 3 mm wide strip of silica gel using a ruler and razor blade. The origin is placed 8 cm from both cut edges (see diagram). In this way distortion due to edge effects is minimized. The prepared plates can be stored at 100°C until required since they are robust enough to be stacked on top of each other.



0.5 µl of emulsifier solution is spotted at the origin in as small a spot as possible. The spot is dried at 100°C for 10 minutes. The plates can be bent so that they can be run in a large glass jar. About 25 ml of solvent is needed and development takes about 20 minutes each way. The plates should be removed as soon as the solvent front has reached the top. The first solvent is removed at 105°C in vacuo for 10 minutes before running in the second solvent. The second solvent also must be removed under the same conditions otherwise it interferes with spot location. The spots are located by spraying with a 0.02% dibromofluorescein solution in 96 % ethanol until pale orange. Dry briefly at 100°C and view under U.V. light. It often helps to respray the plate lightly and allow to dry under the UV. The plates can be written on with soft pencil and copied on a photocopier.

INTERPRETATION

Of the emulsifiers glyceryl monostearate, distilled monoglyceride, propylene glycol monostearate, sorbitan monostearaete, sorbitan tristearate, polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan tristearate, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides and polyglycerol esters, only glyceryl monostearate and lactic acid esters of monoglycerides are indistinguishable. Many emulsifiers give several spots so that mixtures may be difficult to identify.

The presence of polyoxyethylene emulsifiers can be confirmed by adding ammonium cobaltothiocyanate in 50 percent ethanol to the emulsifier concentrate in chloroform-methanol. A blue colour is produced in the organic layer if polyoxyethylene compounds are present.

It is essential to run standard commercial emulsifier preparations as comparisons. Identification should be regarded as tentative.

SACCHARIN

PRINCIPLE

The saccharin is extracted from the acidified sample, and reacted with phenothiazine to form a coloured material which is extracted into xylene and determined spectrophotometrically.

APPARATUS

1. Separatory funnels.
2. Water bath.
3. Spectrophotometer.

REAGENTS

1. Sulphuric acid, 10%.
2. Diethyl ether.
3. Sodium bicarbonate solution, 1%.
4. Hydrochloric acid.
5. Ethanol, 50% and absolute.
6. Cupric acetate solution, 10%.
7. Phenothiazine solution, 5%.
8. Xylene.
9. Anhydrous sodium sulphate.
10. Saccharin standard solution in ether (0.5 mg/ml).

PROCEDURE

Weigh 50 g sample and add 30 ml water. Acidify with 10% sulphuric acid and add 5 ml excess. Place in a separatory funnel and extract twice with 100 ml portions of diethyl ether. Collect the ether extracts in a second separatory funnel and extract twice with 25 ml portions of 1% sodium bicarbonate. Collect the bicarbonate extracts in a third separatory funnel and discard the ether layer. Acidify the bicarbonate extracts with 10% hydrochloric acid. Extract twice with 30 ml ether. Discard the acid layer and wash the ether extracts with 10 ml distilled water. Transfer to a 100 ml flask and evaporate to a small volume at 40°C.

Transfer the saccharin residue in the flask to a 2 x 16 cm test tube using 5 ml of 50% ethanol. Add 1 ml cupric acetate solution, 1 ml phenothiazine solution, and 3 ml absolute ethanol.

Place the tube and contents in a water bath at 65-70°C for 5 minutes shaking occasionally. Cool and transfer the solution to a 50 ml separatory funnel, using 2 ml of absolute ethanol. Add 5 ml xylene and 15 ml water. Shake vigorously for 5-10 minutes. Separate the xylene layer and dry with 1 gm anhydrous sodium sulphate.

Pipette 1, 2 and 4 ml of the saccharin standard solution into three test tubes. Evaporate to a small volume, add 5 ml of 50% ethanol and continue as with the sample starting with addition of cupric acetate solution.

Determine the absorbance of the sample and three standards in 1 cm cells at 510 nm using xylene as the reference. Prepare a standard curve.

CALCULATION

$$\text{Saccharin ppm} = \frac{w}{50} \times 1000$$

where: w = mg saccharin in sample solution read from the standard curve.

REFERENCE

Tanka, A., Nose, N., Suzuki, T., Kobayashi, S. and Watanabe, A., 1977. Analyst 102, 367-70.

ARTIFICIAL SWEETENERS

PRINCIPLE

The food is extracted with ethyl acetate. The concentrated extract is subjected to thin-layer chromatography on silica gel and the spots visualized. Saccharin, cyclamate, 5-nitro-2-propoxyaniline (P-4000) and p-ethoxyphenylurea (dulcin) are detected. The method is designed primarily for beverages.

APPARATUS

1. Apparatus for thin-layer chromatography.

2. Short-wave (254 nm) UV lamp.

REAGENTS

(Note: Prepare solutions fresh on day of use.)

1. Developing solvent - n-butanol:alcohol:ammonia:water (40:4:1:9, by volume).

2. Chromogenic agents - (1) bromine in CCl_4, 5% by volume (great care must be taken with this reagent as bromine is extremely corrosive and toxic and CCl_4 is very toxic); (2) 0.25% fluorescein in dimethylformamide-alcohol (1+1); (3) 2% N-1-naphthyl-ethylenediamine. 2HCl in alcohol.

3. Standard mixture - 50 mg Ca cyclamate, 10 mg Na saccharin, 4 mg dulcin, and 4 mg P-4000 in 10 ml dilute alcohol (1+1). 5µl = 25 µg cyclamate, 5µg saccharin, 1µg dulcin, and 2µg P-4000. Warm solution to dissolve dulcin if necessary. Avoid skin contact with P-4000.

4. Silica gel. Adsorbosil-1 (Applied Sciences Labs., State College, PA 16801) or silica gel (Merck).

5. Sulphuric acid solution, 50% (v/v).

6. Petroleum ether.

7. Ethyl acetate.

8. Ammonium hydroxide:water:alcohol (5:5:10).

PROCEDURE

Decarbonate a carbonated beverage by repeated shaking and pouring. To 50 ml sample in 125 ml separator, cautiously add 10 ml H_2SO_4 (1+1). Cool, extract with two 50 ml portions petroleum ether (shake gently, but thoroughly) and discard petroleum ether. To the aqueous layer, cautiously add 5 ml 50% NaOH solution (w/w), cool, and extract with two 50 ml portions ethyl acetate. (Use 60 ml for cola samples to prevent emulsions). Filter the extracts through ethyl acetate-washed cotton into a beaker or flask with a pouring lip. Evaporate to 5-10 ml on a steam bath (using an air current) and transfer to a graduated tube. (Do not let the solution evaporate to dryness before transfer. Sweeteners may be difficult to redissolve). Evaporate the solution in the graduated tube to dryness on a steam bath with a current of air. Dilute to 2.5 ml with NH_4OH: water:alcohol (5:5:10) and mix thoroughly. (Any insoluble residue in the tube will not interfere with detection).

Slurry 35 g Adsorbosil with 50 ml water or 30 g silica gel H with 75-80 ml water and apply as a 0.25 mm layer to five 20 x 20 cm plates.

Dry the plates for more than an hour at room temperature. Do not dry in the oven. Do not store in a desiccator. Score the layer 5 mm from each side edge and remove a 5 mm band of adsorbent from the bottom edge of the layer. Use the plates within 36 hours of preparation.

Line the developing tank with adsorbent paper. Pour 25 ml of developing solvent into the tank, wetting the paper. Place a V-shaped trough in the tank and add 25 ml of developing solvent to the trough. (Alternatively, put the developing solvent in the tank to about 1 cm). Place a lid on the tank, seal and let stand for about 1/2 hour to saturate the tank atmosphere.

Mark a TLC plate at the side edges only, 2.5 cm from the bottom to designate a spotting line. Mark a dotted line 10 cm above the spotting line. Spot a total of 5 µl each of standard and sample (Level 1). Dilute sample to 5 ml with NH_4OH:water: ethanol (5:5:10) and spot 5 µl (Level 2). Place spots at least 2 cm apart and 2 cm from the edges. Spot 1 µl at a time and use a warm-air blower to dry the spot between applications to restrict the spot diameter. Use the same technique to spot sample and standard. (Total volume spotted should not be more than 5 µl). Use mixed standard rather than superimposed single standards.

Place the plate in the tank and develop to the 10 cm line (about one hour). Dry the plate in a fume hood until the layer is no longer translucent (about 10 minutes). View under shortwave (254 nm) UV. Outline any fluorescent saccharin spot at R_f about 0.5. (Spot may be crescent-shaped if a large amount of cyclamate is present). In the fume hood, spray chromogenic agents (1) and (2), lightly to moderately, in immediate succession, until the cyclamate standard appears as a pink spot at R_f about 0.3 to 0.4. P-4000 is a brown-pink spot at R_f about 0.85. Spray chromogenic agent (3) on a plate until the background pink fades to light yellow. The contrast of cyclamate and P-4000 improves and at R_f about 0.7 dulcin appears. The dulcin spot may be brown-pink or blue, depending on the condition of spray reagents and the concentration of the sweetener. The plate may be resprayed with chromogenic agent (3) to restore contrast if the pink background reappears.

INTERPRETATION

Certain experimental parameters were found to be critical for optimum results. The moisture content of the TLC plates is very important in obtaining good separation of saccharin and cyclamates. The plates must be freshly prepared and dried without heat or desiccation. Limited studies indicate that commercially-prepared TLC plates or sheets are not suitable.

A number of potential interferences are removed from beverages by the petroleum ether prewash. The aqueous solution must be strongly acidified, otherwise any P-4000 present will be extracted by the petroleum ether. Gentle shaking prevents excessive emulsions. Partial neutralization of the acidity of the aqueous phase prior to ethyl acetate extraction resulted in a higher recovery of dulcin and P-4000.

Specifying a technique for applying the chromogenic sprays is rather difficult. Detection has been accomplished on some plates with light spraying but at other times, heavier spraying was needed. The best practice is to spray until the standards are clearly detected. Occasionally, standards fail to show up on a plate but a repeat experiment with a fresh plate is usually successful.

REFERENCE

Korbelak, T., 1969. Journal of the AOAC 52, 487.

MONOSODIUM GLUTAMATE

PRINCIPLE

The monosodium glutamate is isolated from the sample using an ion exchange resin. The glutamic acid is then titrated potentiometrically.

APPARATUS

1. Chromatographic column - 500 x 22 mm OD tube, 30 ml bed volume with Dowex 50 W - X8 (H^+ form) 100-200 mesh.

2. pH meter.

3. Burette.

REAGENTS

1. Activated Carbon - Darco G-60.

2. Acetone.

3. Hydrochloric acid, 1 volume + water, 2.5 volumes.

4. 0.8 N Hydrochloric acid.

5. 1N Hydrochloric acid.

6. 50% Sodium hydroxide.

7. 0.1 N Sodium hydroxide.

8. Formaldehyde.

PROCEDURE

For products in dry form, reduce about 40g to powder in a mortar and weigh 10 g of sample into a 250 ml beaker. For undiluted condensed soups or canned green beans, homogenise entire undiluted content of can in blender and weigh 20 g of sample into a 250 ml beaker. For consomme type (clear, condensed) soup weigh 20 g into a 250 ml beaker.

Dilute the sample to about 70 ml with water at room temperature and mix until all water-soluble substances are in solution (about 15 minutes). Add 6 g of activated carbon and mix thoroughly. (For products containing starch, also add 60 ml of acetone to precipitate starch and to aid sample solution). Let stand 30 minutes (or overnight). Filter with vacuum through a 60 ml coarse, fritted glass funnel containing an asbestos pad, wash flask and residue with six 25 ml portions of water or (if acetone was added) with six 25 ml portions of acetone-water (1+1).

Collect filtrate and washings in a 400 ml beaker, add 2 drops hydrochloric acid (1 + 2.5) and evaporate on a steam bath to about 40 ml. (Hydrochloric acid prevents conversion of glutamic acid to pyrrolidone carboxylic acid). Quantitatively transfer to a 50 ml volumetric flask, dilute to volume with water and mix.

Transfer 25 ml of extract to prepared column and adjust flow to about 0.5 ml/minute. After all the solution enters the resin, wash column wall with about 10 ml of water. Let wash pass into the resin. Add 120 ml 0.8 N hydrochloric acid and maintain flow rate (0.8 N HCL will elute any serine, threonine and aspartic acid). After all the 0.8 N

hydrochloric acid passes into the resin, add 170 ml of 1 N hydrochloric acid and adjust the flow rate to between 25 and 30 drops/min to elute glutamic acid. Collect eluate in a 400 ml beaker. (Any glycine present will elute after 200 ml of 1N hydrochloric acid). Nearly neutralise with 50% sodium hydroxide. Neutralize 25 ml of 37% formaldehyde to pH 7 with 0.1 N sodium hydroxide and adjust potentiometrically to pH 7 with 0.1 N sodium hydroxide and add to prepared neutral sample. Mix for 10 minutes on a magnetic stirrer and titrate potentiometrically to pH 8.9 with 0.1 N sodium hydroxide.

Determine blank by titrating to pH 8.9 a mixture of 25 ml of neutralised formaldehyde and 170 ml of neutralised 1N hydrochloric acid.

CALCULATION

% Glutamic acid = (S-B) x N x 0.147 x 100/W

Where: S = ml NaOH used to titrate sample, B = ml NaOH used to titrate blank, N = normality of NaOH and W = weight of sample.

% M.S.G. = % Glutamic acid x 1.15 (anhydrous).

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7. CONTAMINANT RESIDUE METHODS

7.1 PESTICIDES

There are hundreds of pesticides in use throughout the world. However, most countries have specified by law or regulation which pesticides may be used on their foods. In this context, the term "pesticides" means industrial chemicals used to kill insects (insecticides), destroy unwanted plants (herbicides) and to prevent mould and mildew (fungicides). By far the greatest amount of 'pesticides' used are insecticides; so the analytical methods given here will target these compounds and fungicides to a lesser extent.

In most cases, the laboratory will not have data on which pesticides were used on a particular food sample. This is even more true for imported foods. Therefore, there must be close cooperation between the laboratory and the inspectorate staff. For domestically grown foods, the inspectorate should be able to determine at least which pesticides are being used in the harvest area. This information would provide a starting point for the laboratory analysis.

For the purposes of residue analysis, pesticides may be divided into four groups - chlorinated hydrocarbon compounds to which the electron-capture detector responds, organo-phosphorus compounds for which the specific phosphorus detectors are the most appropriate, carbothioates (thio-carbamates, often of metals such as zinc and manganese), and a miscellaneous group which includes compounds of diverse chemical types such as quintozene and dichloropropionic acid. The term chlorinated hydrocarbon is intended here to cover only the commonly encountered organo-chlorine insecticides such as DDT and its derivatives, the various isomers of BHC and the chlorinated polycyclic compounds such as Aldrin and Heptachlor.

The initial pesticide residue analysis of a food is by necessity a broad screening examination for compounds of a given type (i.e. organo-chlorines). Such screening methods are called "multiresidue" procedures. A multiresidue analysis can be discussed in four distinct steps:

1. Preparation

The initial preparation of the food sample is as important as any of the other residue analysis steps. Two things are critical. First, remove and discard any inedible portions. This could be an outer skin or pod, an inner seed or pit, or anything else that is obviously inedible. Never discard portions which are merely undesirable (such as the wilted outer leaves of a leafy vegetable). Next, chop or grind, and mix so that the portion taken for analysis is as representative as possible.

2. Extraction

In this step, the pesticide residues and soluble food material are extracted into an appropriate solvent. The insoluble material (such as fibre or cellulose) is removed by filtration. The selection of the solvent is crucial. Pesticides range widely in polarity from the non-polar (fat or wax soluble) chlorinated hydrocarbons to the polar (more water soluble) organo-phosphates or carbamates. The solvent used must bridge the gap between these polarities and be able to simultaneously extract all residues. Acetone has been found to work very well in this respect. A second problem of the extraction process is that the solvent must physically contact the site of the residue. If the residue was only on the food outer surface, then a simple washing or tumbling with the solvent would suffice. In practice, however, the residue must be assumed to have been incorporated into the food matrix. It is therefore necessary to blend the food with the solvent to gain the intimate contact needed to ensure quantitative extraction.

3. Isolation

This is often referred to as the "clean-up" in published residue methods. It is where the solubilized pesticide residues are isolated and the other soluble interfering materials are removed. There are several ways to do this, which include partitioning between solvents, adsorption on a solid material or outright destruction of the interferences. All multiresidue methods employ some form of partitioning. Most also use adsorption as the preferred means to remove interferences. Only a few employ drastic destruction methods as the residues must be able to survive and not many are that chemically stable.

4. Detection

The technique that offers the most versatility and greatest sensitivity for pesticide residue analysis is gas chromatography. High performance liquid chromatography has some applicability to residues which are heat sensitive and degrade during gas chromatographic analysis. Thin-layer and paper chromatography are most useful for confirmation of identity after the residue has been tentatively identified by gas chromatography. Some common gas chromatograph detectors are:

a. Electron Capture

The advantages of this detector are its low maintenance requirements and high sensitivity for halogenated compounds. However, it is also responsive to compounds that have electronegative groups such as carbonyls or double bonds. Lovelock(1) notes that if the detector is used in a dc mode and not in a pulsed mode, its performance can also be affected by nonelectronegative compounds that survive the isolation procedure.

b. Flame Photometric

This detector is relatively specific to either organo-phosphorus or organo-sulfur compounds, depending on the light filter that is used. The detector has a linear response range of about four decades of concentration for organo-phosphates but has a response that is proportional to the square of the concentration of organo-sulphur compounds. It is therefore most useful for the determination of organo-phosphorus compounds. However, care should be exercised in the interpretation of the chromatograms of samples with a high sulphur background when organo-phosphorus compounds are being determined. The presence of large amounts of sulphur cannot be effectively filtered out and could therefore produce peaks that may be misinterpreted as phosphorus compounds.

c. Alkali Flame

There are several commercial versions of the alkali flame detectors. Some use different alkali salts in the detector, and one does not use a flame at all but heats an alkali bead to the desired operating temperature. The detectors are more responsive to compounds that contain phosphorus or nitrogen, with a greater sensitivity for phosphorus. Their performance can be affected to varying degrees, by residual amounts of halogenated solvents in the sample extract, depending on the commercial detector used.

d. Electroconductivity

Design improvements by Hall(2) and Anderson(3) have resulted in electroconductivity detectors capable of determining nanograms of organo-nitrogen compounds and subnanogram amounts of organo-sulphur and organo-halogen compounds. The effluent from the gas chromatographic column is either oxidized or reduced to produce a species that becomes ionic when dissolved in a suitable solvent. Organo-nitrogen compounds are reduced to ammonia. Organo-sulphur compounds are oxidized to the sulphur oxides. Organo-halogen compounds can be oxidized or reduced to form the hydrogen halides. The specificity of the

detectors is attained by the use of scrubbers that remove other reaction by-products and by the selection of an appropriate conducting solvent. The detector is very reliable and usual maintenance generally consists only of replacement of the conducting solvent every few weeks.

Residue analysis is complex but is further complicated by the fact that pesticides are often degraded by oxidation or hydrolysis. This can occur in the environment by the action of sunlight and air, and in plants or animals by enzymatic and other metabolic processes. The degradation products are often generically referred to as "metabolites". Many times the metabolite is less toxic than the original compound and may be biologically inactive. In some cases, however, the metabolites are equally or even more toxic than the original. An example of the latter is Heptachlor Epoxide which is considerably more toxic than Heptachlor. The residue analyst must be aware of the more commonly found metabolites which do have biological toxicity (such as DDE and DDD from DDT).

Another complicating factor in residue analysis is the potential presence of environmental contaminants which are not pesticides, but which may interfere in analysis. The best examples are polychlorinated biphenyls (PCB), chemicals commonly used as a heat exchange fluid for cooling transformers or processing machinery. PCBS are very stable and once in the environment will stay there indefinitely. They are a mixture of chlorinated compounds and usually appear as a series of peaks on a gas chromatogram.

Sometimes peaks are detected which can best be described as natural artifacts. They are sometimes referred to as "crop peaks" and consist of some natural food ingredient which is not removed by the isolation or clean-up process.

The possible presence of metabolites, contaminants and crop peaks underscore the need to confirm the identity of any pesticide residue found. Thin-layer and paper chromatography can be used, but may not be sensitive enough to confirm very low level residues. In such cases it is usually sufficient to gas chromatograph the sample and standard on two or three columns having differing polarities. If the sample and standard have the same relative retention time on all of the columns, it can be assumed that they are probably the same.

Make sure that the pesticide standards used for residue analysis have not undergone degradation themselves while in storage. Chlorinated hydrocarbons like DDT and BHC are generally quite stable, but many organo-phosphates and carbamates are unstable and need refrigerated storage as well as frequent checks on their stability. Also remember that dilute working standards are even more prone to degradation, as well as concentration by solvent evaporation. Make fresh working standards frequently.

The sensitivity of modern GLC detectors requires extreme care be taken to use only the purest solvents, reagents and gases. Nitrogen carrier gas can be purified by inserting a molecular sieve between the cylinder and the instrument. Solvents, however, are purified by redistillation (from glass). A quick check of a solvent's suitability is to evaporate 300 ml to 5 ml and inject 5 µl of this into a gas chromatograph. There should be no extraneous peaks and the baseline should not raise more than 1 mm for up to one hour.

RESIDUES IN FRUITS AND VEGETABLES

PRINCIPLE

This method is applicable to organo-chlorine, organo-phosphorus and organo-nitrogen pesticides, as well as a few hydrocarbon pesticides. The pesticide residues are extracted into acetone and after partitioning, the organo-phosphorous and organo-nitrogen pesticides are determined directly using an alkali-flame GLC detector. Interferences are removed using Florisil before determining organo-chlorine compounds using electron capture detection.

APPARATUS

1. High speed blender - Waring blender or equivalent. (explosion proof).

2. Chromatographic tube with stopcock, 22 mm x 300-400 mm.

3. Kuderna-Danish (KD) concentrator, 500 ml with 10 or 15 ml graduated receiving tubes and Snyder column.

4. Separatory funnels, 1 L.

5. Gas chromatograph with electron capture and flame photometric or alkali-flame detectors, and nitrogen carrier gas.

6. GLC Columns, glass, 6 feet (or 2 m) x 4 mm ID, packed with the following liquid loads on 80-100 mesh Chromosorb W (HP): (nitrogen flow rate and column temperature also indicated).

   a. 2% DEGS (Diethyleneglycolsuccinate) at 165°C and 60 ml/min N_2.

   b. 3% OV-101 at 200°C and 120 ml/min.

   c. 10% OV-101 at 200°C and 120 ml/min.

   d. 10% OV-101 and 15% OV-210 at 200°C and 120 ml/min.

7. Steam bath.

REAGENTS

1. Acetone

2. Methylene chloride

3. Petroleum ether

4. Ethyl ether

(Note: all solvents must be distilled from glass or equivalent).

5. Florisil, 60-100 mesh - activate by heating at 130°C for 5 hours.

6. Sodium sulphate, anhydrous, granular.

7. Glass wool - rinse with acetone and ethanol several times and dry - washed glass wool will be somewhat brittle.

8. Sodium chloride, granular.

PROCEDURE

Chop or blend fruits and vegetables and mix thoroughly. Weigh 100g chopped or blended sample into high-speed blender jar, add 200 ml acetone, and blend 1 min at high speed. Do not add Celite. Filter with suction through 12 cm Buchner funnel fitted with sharkskin paper, collecting extract in 500 ml suction flask.

Place 80 ml sample extract in 1 L separatory funnel, and add 100 ml pet. ether and 100 ml $MeCl_2$. Shake vigorously 1 min. Transfer lower aqueous layer to second 1 L separatory funnel. Dry upper organic layer of first separatory funnel by passing through about 3.4 cm Na_2SO_4 supported on washed glass wool in a 10 cm funnel, collecting in 500 ml Kuderna-Danish concentrator fitted with receiving tube. To separatory funnel with aqueous phase add 7g NaCl and shake vigorously 30 sec until most of the NaCl is dissolved. Add 100 ml $MeCl_2$, shake 1 min, and dry lower organic phase through same Na_2SO_4. Extract aqueous phase with additional 100 ml $MeCl_2$ and dry as above. Rinse Na_2SO_4 with ca 50 ml $MeCl_2$. Add boiling chips and attach Snyder column on Kuderna-Danish concentrator and start evaporation slowly by placing only receiver tube into steam. After 100-150 ml has evaporated, the concentrator may be exposed to more steam. When liquid level in hot concentrator tube is ca 2 ml, add 10 ml acetone and reconcentrate to ca 2 ml. Repeat above concentration with two additional 10 ml portions of acetone. Do not allow solution to go to dryness.

Analysis of Organo-phosphorus and Organo-nitrogen Compounds

Adjust the volume in the concentrator tube to 7 ml with acetone. Inject 5 µl of this solution (equivalent to about 20 mg sample) into gas chromatograph using phosphorus detector and column conditions (a), (b) or (c). Column (d) may be used to confirm residue identity.

Analysis of Organo-chlorine Compounds

Dilute 7 ml solution in concentrator tube to 10 ml with acetone. Transfer to a 100 ml glass-stoppered graduated cylinder using petroleum ether to rinse. Dilute to 100 ml with petroleum ether, stopper and mix well.

Prepare a Florisil column by placing 10 cm activated Florisil in a chromatographic tube. Top with 1 cm anhydrous sodium sulphate. Place a KD concentrator under the column to collect the eluate.

Wet Florisil with 50 ml pet. ether. Elute at about 5 ml/min. Add entire sample solution (100 ml) to Florisil and continue elution. When the top of the sample solution enters the sodium sulphate, add 200 ml 15% ethyl ether in pet. ether. Elute until the liquid has drained from the column.

Evaporate and concentrate to 4 ml. Inject 5 µl (equivalent to about 34 mg sample) into a gas chromatograph using electron capture detector and column conditions (b) or (c). Columns (a) or (d) may be used for confirmation.

Analysis of Hydrocarbon Compounds

Inject 5 µl (about 34 mg sample equivalent) into a gas chromatograph using flame ionization detector and column conditions (c).

CALCULATION

$$\frac{\text{mg sample}}{\mu\text{l injected}} = \frac{(100)(80)}{(200 + W - 10)(V)}$$

where: 100 = initial g sample
80 = ml taken for analysis
200 = ml acetone
W = ml water in 100 g sample
10 = acetone/water concentration factor
V = ml of final solution (7 or 4)

(Note: As a first approximation, W = 85 for most fruits and vegetables. Water content of raw produce may be found in food composition tables.)

REFERENCE

Luke, M.A., Froberg, J.E. and Masumoto, H.T., 1975. Journal of the Association of Official Analytical Chemists 58, 1020-1026.

RESIDUES IN MILK AND OILSEEDS

PRINCIPLE

Milk is blended with aluminium oxide and acetonitrile/water. The fat is adsorbed on the Al_2O_3 and fat-soluble pesticides partition from the fat, directly into the CH_3CN. Oilseeds are treated the same way after grinding. The method is applicable to organo-chlorine pesticides. It is very rapid compared to procedures where the fat is first extracted and then examined for residues.

APPARATUS

1. High speed blender, Waring or equivalent (explosion proof).

2. Food grinder with 2 mm screen.

3. Separatory funnels, 1 L.

4. Kuderna-Danish (KD) concentrator, 500 ml, with 15 ml graduated receiving tube, and a Snyder column.

5. Chromatographic tube with stopcock, 22 mm x 300 - 400 mm.

6. Gas chromatograph with electron capture detector and nitrogen carrier gas at 120 ml/min.

7. GLC column, glass, 6 feet (or 2 m) x 4 mm ID, packed with:

   a. 3% OV-17 on 80-100 mesh Chromosorb W(HP)

   b. 2% OV-101 on 100-120 mesh Chromosorb W(HP)

8. Steam bath.

REAGENTS

1. Acetonitrile.

2. Petroleum ether.

3. Ethyl ether.

(Note: All solvents must be distilled from glass or equivalent).

4. Aluminium oxide, 80-200 mesh - for each g, wash with 10 ml 90% ethanol, then 10 ml hexane, drain and dry on a steam bath.

5. Florisil, 60-100 mesh - activate by heating at 130°C for 5 hours.

6. Sodium sulphate, anhydrous, granular - heat to 600°C for 1 hour, cool and store in desiccator.

7. Glass wool - rinse with ethanol several times and dry.

8. Saturated sodium chloride solution.

PROCEDURE

Analysis of Milk (Containing 4% or Less Fat)

Weigh 50 g milk into blender cup. Add 20 g aluminium oxide, 25 ml distilled water and 280 ml acetonitrile. Blend 2 min at high speed. Wait for solids to settle, then filter supernatant liquid using vacuum.

Measure 250 ml of filtrate and transfer to 1 L separatory funnel. Add 100 ml pet. ether and shake 30 sec. Add 10 ml saturated NaCl solution and 500 ml water. Shake 1 min and let layers separate.

Drain lower aqueous layer into second 1 L separatory funnel. Add 100 ml pet. ether to second funnel and shake 1 min. Let layers separate and drain and discard lower aqueous layer.

Combine pet. ether layer in second funnel with pet. ether in the first. Wash the combined pet. ether with two successive 100 ml portions water. Drain and discard the water washes.

Dry the pet. ether by passing through anhydrous Na_2SO_4 and collect in a KD concentrator. Concentrate to 10 ml.

Prepare a Florisil column by placing 10 cm Florisil in a chromatographic tube. Top with 1 cm anhydrous Na_2SO_4. Place a KD concentrator under the column to collect the eluate.

Wet Florisil with 50 ml pet. ether. Elute at about 5 ml/min. Add all of sample concentrate (10 ml) using pet. ether to rinse. When the top of the sample solution enters the sodium sulphate, add 200 ml 6% ethyl ether in pet. ether. Elute until the liquid has drained from the column. Place a second KD concentrator under the column and elute with 200 ml 15% ethyl ether in pet. ether.

Concentrate both individually, on a steam bath to 4 ml. Inject 5 µl (equivalent to about 45 mg of sample) of each into a gas chromatograph using column (a) or (b). (One could be used for analysis and the other for confirmation). The use of 6 and 15% elutions is to separate the pesticides Dieldrin and Endrin from DDE and Aldrin. A single elution of 15% ethyl ether could be used, to elute all pesticides at once.

Analysis of Oilseeds

Grind the oilseeds to pass a 2 mm screen three times. Weigh a portion containing not more than 2 g fat into a blender cup. Add 20 g Al_2O_3 and 350 ml of acetonitrile-water (80+20).

Continue as above for milk starting with "...Blend 2 min...".

CALCULATIONS

For milk:

$$\frac{\text{mg sample}}{\mu\text{l injected}} = \frac{(50)(250)}{(344)(V)}$$

where:
- 50 = g sample
- 250 = ml taken for analysis
- 344 = CH_3CN/water factor
- V = ml of final solution

For oilseeds:

$$\frac{\text{mg sample}}{\mu\text{l injected}} = \frac{(W)(250)}{(350)(V)}$$

where: W = g sample
250 = ml taken for analysis
350 = CH_3CN/water factor
V = ml of final solution

REFERENCE

Luke, M.A. and Doose, G.M., 1984. Bulletin of Environmental Contamination and Toxicology 32, 651-656.

RESIDUES IN DRY, LOW-FAT FOODS

PRINCIPLE

"Dry" products are defined as having less than 10% water, such as dried vegetables, spices and pulses. The problem with analysis of such products has been the difficulty of extraction of the residues from the sample matrix. This difficulty was solved by use of a water/acetone mixture. The method is designed for relatively low-fat food products but can be used for foods containing up to 10% fat. The Florisil isolation step must be used when the food contains above 2-3% fat.

APPARATUS

1. High speed blender - Waring blender or equivalent (explosion proof).

2. Separatory funnel, 1 L.

3. Kuderna-Danish (KD) concentrator 500 ml with 10 ml graduated receiving tube and Snyder column.

4. Chromatographic tube with stopcock, 22 mm x 300-400 mm.

5. Gas chromatograph with electron capture and flame photometric or alkali flame detectors and nitrogen carrier gas.

6. GLC column, glass, 6 feet (or 2 m) x 4 mm ID packed with 3% OV-101 on 80-100 mesh chromosorb W(AW). Nitrogen flow rate of 120 ml/min and column temperature of 200°C.

REAGENTS

1. Acetone

2. Methylene chloride

3. Petroleum ether

4. Ethyl ether

(Note: all solvents must be distilled from glass or equivalent.)

5. Activated carbon

6. Celite 545 (diatomaceous earth)

7. Magnesium oxide, 200 mesh, adsorptive grade

8. Florisil, 60-100 mesh - activate by heating at 130°C for 5 hours.

9. Sodium sulphate, anhydrous, granular.

10. Sodium chloride

PROCEDURE

Grind sample to pass 20 mesh. Weigh 15 g into blender cup and add 350 ml acetone + water mixture (65+35). Blend at high speed 2 min. Filter and transfer 80 ml filtrate to 1 L separatory funnel.

Add 100 ml methylene chloride and 100 ml petroleum ether. Shake vigorously 1 min. Allow layers to separate and transfer the lower aqueous layer to a second 1 L funnel.

Dry the upper organic layer by passing through sodium sulphate and collect in a KD concentrator.

Add 7 g sodium chloride to the second funnel. Extract with 2/100 ml portions of methylene chloride by shaking 1 min. Dry each lower organic phase by passing through sodium sulphate and collect all in the KD concentrator.

Rinse the sodium sulphate with 50 ml methylene chloride and add to the KD. Concentrate the combined extracts to 2 ml. Add 2/10 ml portions acetone and reconcentrate to 2 ml after each addition.

This extract can be injected directly into the gas chromatograph, or can be further cleaned using the carbon or Florisil columns below.

Carbon Column

Prepare by placing 2.5 cm Celite in a column. Then add 6 g of a carbon mixture containing carbon + Celite + magnesium oxide (1 + 4 + 2). Tamp down firmly and wash with 25 ml methylene chloride. Use air pressure to elute through the column. Discard the wash and place a KD under the column.

Add the sample extract to the column using small portions of methylene chloride to transfer. When the sample solution enters the column, elute with 200 ml acetone + methylene chloride (2 + 1). Concentrate as above.

Florisil Column

Prepare and use as described in the method, "Residues in Milk and Oilseeds".

CALCULATIONS

$$\frac{\text{mg sample}}{\mu\text{l injected}} = \frac{(15)\ (80)}{(350)\ (V)}$$

where:
- 15 = g sample
- 80 = ml taken for analysis
- 350 = acetone/water
- V = ml of final solution

REFERENCE

Luke, M.A. and Doose, G.M., 1983. Bulletin of Environmental Contamination and Toxicology, 30, 110-116.

RESIDUES IN FATTY FOODS

PRINCIPLE

This procedure is only applicable to fat-soluble, non-polar pesticides. The method involves first extracting the fat, then isolating the pesticide residues, by partitioning and adsorption chromatography.

APPARATUS

1. High speed blender - Waring blender or equivalent (explosion-proof)

2. Separatory funnels, 125 ml and 1 L.

3. Kuderna-Danish (KD) concentrator, 500 ml with 10 ml receiving tube and Snyder column.

4. Chromatographic tube with stopcock, 22 mm x 300-400 mm.

5. Gas chromatograph with electron capture detector and nitrogen carrier gas.

6. GLC column, glass, 6 feet (or 2 m) x 4 mm ID, packed with 3% OV-101 on 80-100 mesh Chromosorb W (AW). Nitrogen flow rate 120 ml/min and column temperature of 200°C.

7. Steam bath

8. Water bath

REAGENTS

1. Acetonitrile

2. Petroleum ether

3. Ethyl ether

(Note: all solvents must be distilled from glass or equivalent.)

4. Sodium sulphate, anhydrous, granular

5. Ethanol

6. Florisil, 60-100 mesh - activate by heating at 130°C for 5 hours.

7. Sodium (or potassium) oxalate

8. Sodium chloride

9. Sodium hydroxide

PROCEDURE

Isolate fat (at least 3 g) as follows:

Animal and Vegetable fats and oils: If solid, warm until liquid and filter through dry filter paper.

Butter: Warm to about 50°C until the fat separates and decant the fat through a dry filter.

Cheese: Place 25-100 g (sufficient to provide 3 g fat) of diced sample, about 2 g of oxalate and 100 ml ethanol in a high-speed blender and blend 2-3 minutes. (If experience with the product indicates that emulsions will not be broken by centrifuging, add 1 ml water/2 g sample before blending). Pour into a 500 ml centrifuge bottle. Add 50 ml of ethyl ether and shake vigorously 1 minute. Then add 50 ml of petroleum ether and shake vigorously 1 minute. Centrifuge about 5 min at 1500 rpm. Siphon off the solvent layer using a wash bottle device prepared as follows: Use tube similar to delivery tube of ordinary wash bottle but with intake end bent up into U-shape in opposite direction to outlet end, with opening 6-12 mm higher than bottom of U, cut off horizontally. (Avoid excessive constriction when bending). Set delivery tube loosely enough in stopper that it can be raised or lowered. In operating, adjust opening of U bend to about 3 mm above surface of aqueous layer and blow ether layer off by gently blowing through mouthpiece tube inserted in adjacent hole in stopper. Siphon into a 1 L separator containing 500-600 ml of water and 30 ml of saturated salt solution. Re-extract the aqueous residue twice, shaking vigorously with 50 ml portions of ethyl ether-petroleum ether (1 + 1). Centrifuge and siphon off the solvent layer into the separator after each extraction. Mix the combined extracts and water cautiously. Drain and discard the water layer. Rewash the solvent layer twice with 100 ml portions of water, discarding the water each time. (If emulsions form, add about 5 ml of saturated salt solution to the solvent layer or include it with the water wash). Pass the ether solution through a column of anhydrous sodium sulphate 50 mm high in a tube and collect the eluate in a 400 ml beaker. Wash the column with small portions of petroleum ether and evaporate the solvent from the combined extracts on a water-bath at 100°C with the assistance of a current of air.

Fish: Weigh 25-50 g thoroughly ground and mixed sample into a high-speed blender. (If the fat content is known or can be estimated, adjust the sample size so that a maximum of about 3 g of fat will be extracted). Add 100 g of anhydrous sodium sulphate to combine with water present and disintegrate the sample. Alternately blend and mix with a spatula until the sample and sulphate are well mixed. Scrape down the sides of the blender jar and break up caked material with a spatula. Add 150 ml of petroleum ether and blend at high speed for 2 minutes. Decant the supernatant petroleum ether through a 12 cm Buchner funnel fitted with 2 sharkskin papers into a 500 ml suction flask. Scrape down the sides of the blender jar and break up the caked material with a spatula. Re-extract the residue in the blender jar with two 100 ml portions of petroleum ether, blending for 2 min each time. (After blending 1 min, stop the blender, scrape down the sides of the jar and break up caked material with a spatula, then continue blending for 1 min). Scrape down the sides of the blender jar and break up caked material between extractions. Decant the supernatant petroleum ether from repeated blendings through the Buchner funnel and combine with the first extract. After the last blending, transfer the residue from the blender jar to the Buchner funnel and rinse the blender jar and material in the Buchner funnel with several portions of petroleum ether. Pour the combined extracts through a 40 mm column of anhydrous sodium sulphate in a tube, and collect the eluate in a 500 ml KD concentrator with a plain collection tube. Wash the flask and column with small portions of petroleum ether and evaporate most of the petroleum ether from the combined extracts and rinses. Transfer the concentrated fat extract to a tared beaker using small amounts of petroleum ether. Evaporate the petroleum ether on a water-bath at 100°C under a current of dry air.

Other fatty foods: weigh into blender jar amount estimated to provide ca 3 g of fat. (Refer to tables of average composition, if necessary.) In separate container mix amount of sodium sulphate equal to 2.5 x estimated weight of water in sample with 100 ml petroleum ether, and transfer to blender jar. Mix at medium speed for 3 min. Allow solids to settle and decant petroleum ether through medium porosity filter paper into tared 500 ml erlenmeyer flask to which a few boiling chips had been added before weighing. Add 100 ml petroleum ether to residue in blender jar, mix at medium speed for 1 min, allow solids to settle and decant petroleum ether through filter to combine with above filtrate. Transfer solid residue in blender jar to filter paper, fold paper over solids, and squeeze paper gently against side of funnel with spatula to recover as much solvent as possible. Evaporate petroleum ether on a water bath. Dry flask and weigh flask plus contents. Determine amount of fat by subtracting tare weight of flask.

Determine the percent fat in the food from the weight of the extracted fat. (This will be used later in the final calculation.)

Weigh 3 g of fat into a 125 ml separator and add petroleum ether so that the total volume of fat and petroleum ether is 15 ml. Add 30 ml of acetonitrile saturated with petroleum ether. Shake vigorously 1 min, allow the layers to separate and drain the acetonitrile into a 1 L separator containing 650 ml water, 40 ml saturated salt solution and 100 ml of petroleum ether. Extract the remaining petroleum ether in the 125 ml separator with 3 additional 30 ml portions of acetonitrile saturated with petroleum ether, shaking vigorously 1 min each time. Combine all the extracts in the 1 L separator.

Hold the 1 L separator in a horizontal position and mix thoroughly 30-45 seconds. Let the layers separate and drain the aqueous phase into a second 1 L separator. Add 100 ml of petroleum ether to the second separator, shake vigorously 15 seconds and let the layers separate. Discard the aqueous phase, combine the petroleum ether with that in the first separator and wash with two 100 ml portions of water. Discard the washings and draw off the petroleum ether layer through a 50 mm column of anhydrous sodium sulphate into a 500 ml KD concentrator. Rinse the separator and then the column with three approximately 10 ml portions of petroleum ether. Evaporate the combined extract and washings to about 10 ml.

Prepare a columm of Florisil 10 cm high (after settling) and topped with about 1 cm of anhydrous sodium sulphate. Wet the column with 40-50 ml of petroleum ether. Place the KD evaporator (with a graduated collection tube under the column) to receive the eluate. Transfer the petroleum ether concentrate to the column and let it pass through at 5 ml/min. Rinse containers with two approximately 5 ml portions of petroleum ether, pour the rinsings on to the column, rinse the walls of the tube with additional small portions of petroleum ether. Elute at about 5 ml/min with 200 ml of 6% ethyl ether in pet. ether. Change receivers and elute with 200 ml of 15% ethyl ether in pet. ether.

Concentrate each eluate to a suitable definite volume in the KD concentrators. The 6% eluate contains chlorinated pesticides such as Aldrin, BHC, DDE, DDD, o,p'- and p,p'-DDT, Heptachlor, Heptachlor Epoxide, Lindane, Methoxychlor, Mirex and Perthane and industrial chemicals such as polychlorinated biphenyls (PCB). It is usually suitable for GLC directly. If further clean-up is necessary, repeat the Florisil clean-up using a new column. The 15% eluate contains the chlorinated pesticides Dieldrin and Endrin. In those cases where the 15% eluate must be further cleaned, destruction of the fatty material can be accomplished by saponification, as follows:

Transfer the concentrated eluate to an 125 ml glass-stoppered flask, rinsing with petroleum ether and evaporate just to dryness. Add 20 ml of 2% ethanolic sodium hydroxide and reflux 30 minutes under an air condenser. Transfer to an 125 ml separator and rinse the flask with three 10 ml portions of petroleum ether, transfering each to the separator. Add 20 ml of water and shake vigorously. Drain the aqueous layer into a second separator containing 20 ml of petroleum ether. Shake vigorously, allow to separate, discard the aqueous layer and add petroleum ether to the first separator. Wash the combined petroleum ether extracts with three 20 ml portions of aqueous ethanol (1 + 1). (If the initial aqueous alcohol wash causes heavy emulsions, use water only for additional washes.) Discard the wash. Dry the petroleum ether layer through a 50 mm column of anhydrous sodium sulphate rinsing with petroleum ether. Concentrate the solution using a KD concentrator, to suitable volume (2-4 ml) for gas chromatography.

Inject 5 µl of the final cleaned extract into the gas chromatograph and compare to known standards.

CALCULATIONS

On a **fat basis:**

$$\frac{\text{mg fat}}{\mu\text{l injected}} = \frac{(W)}{(V)}$$

where:

W = g fat taken for analysis
V = ml of final solution

On a **whole food basis:**

$$\frac{\text{mg sample}}{\mu\text{l injected}} = \frac{(W)}{(V)(\%)}$$

where:

W = g fat taken for analysis
V = ml of final solution
% = percent fat in the sample expressed as a decimal (i.e. 12% fat = 0.12)

REFERENCES

Pesticide Analytical Manual, Vol. I, U.S. Food and Drug Administration, Washington, D.C.

Official Methods of Analysis of the AOAC, 1984, 29.001-.002, 29.008-.018.

RESIDUE IDENTITY CONFIRMATION
(Using Thin-Layer Chromatography)

PRINCIPLE

See 5.2 of this Manual for a general discussion of TLC technique, including preparation of plates.

APPARATUS

1. TLC apparatus including glass plates, developing tanks, spotting pipettes and TLC spray flasks.

2. Forced draft oven.

3. UV viewing cabinet.

REAGENTS

1. Aluminium oxide, neutral G or equivalent.

2. Developing solvents for chlorinated pesticides: (a) n-Heptane (b) n-Heptane containing 2% acetone.

3. Chromogenic agent for chlorinated pesticides: Dissolve 0.100 g silver nitrate in 1 ml water, add 20 ml of 2-phenoxyethanol, dilute to 200 ml with acetone, add a very small drop of 30% H_2O_2 and mix. Store in dark overnight and decant into a spray bottle. Discard after 4 days.

4. Developing solvents for phosphated pesticides: (a) Immobile; 15 or 20% N,N-dimethylformamide (DMF) in ether. Dilute 75 or 100 ml of DMF to 500 ml with ether and mix. (b) Mobile; methylcyclohexane.

5. Chromogenic agents for phosphated pesticides: (a) Stock dye solution; dissolve 1 g tetrabromophenolphthalein ethyl ester in 100 ml acetone. (b) Dye solution; dilute 10 ml of the stock dye solution (a) to 50 ml with acetone. (c) Silver nitrate solution; dissolve 0.5g in 25 ml water and dilute to 100 ml with acetone. (d) Citric acid solution; dissolve 5 g of granular citric acid in 50 ml of water and dilute to 100 ml with acetone.

PROCEDURE

Preparation of TLC Plates

Prepare plates as described in Chapter 5.2 of this Manual, using a slurry of 30 g aluminium oxide and 50 ml water. When making the slurry, be sure to shake only moderately for 45 seconds. Violent shaking produces bubbles, resulting in "pock-marked" layers. (Note: Suspensions that contain adsorbents with binders set rapidly and the entire operation from preparation of slurry to final coating must be completed within 2 minutes.) This amount is sufficient for about five plates.

Let the plates air dry for 15 min, then 30 min in a forced-draft oven at 80°C. Cool the plates in a dry area. Examine the plates carefully in transmitted and reflected light for imperfections or irregularities in coating. Discard any plates on which the layer shows extensive rippling or mottling.

Pre-washing of Plates

Scrape 1 cm of adsorbent off the edge of the plate with a razor blade. Pour 15 ml of 50% aqueous acetone into a metal trough inside a chromatographic tank. Cut out a 2 x 20 cm strip of Whatman No. 1 filter paper, wet with solvent and place over the scraped-off area with 6 mm of the paper overlapping the adsorbent layer. Place the plate in the chromatographic tank, seal the tank with masking tape and develop the plate with 50% aqueous acetone to within 4 cm of the top (75-90 minutes). Remove the plate from the tank, detach the filter paper wick, invert the plate and dry in a fume hood for 5 minutes. Dry the plate for 45 minutes at 80°C. Remove the plate from the oven, cool and store in a desiccator. Use the prepared plates within 1 week.

Sample Application

Mark the plate with a pencil on both sides 4 cm from the bottom. The imaginary line between the two points indicates sample spotting or origin line. Draw a line, removing the layer completely across the plate 14 cm from the bottom edge. This line represents the solvent front after development. On the lower edge of adsorbent starting 2 cm in from the left side of the plate make 18 marks with a pencil at 1 cm intervals. (Fewer marks at longer intervals may be used, if desired.) The marks serve as horizontal guides to sample application. Identity of samples and standards may be etched into adsorbent layer directly above these marks at the top of the plate.

Use the shadow cast by a strong light source on a straight edge supported about 2 cm above the plate to show a straight line across the paper. Align the shadow on the two 4 cm marks on either edge of the plate. The line of the shadow and the 18 marks serve as vertical and horizontal guides respectively for application of the sample.

For optimum semi-quantitative determination, spot portions of the samples as follows:

For Chlorinated pesticides: Adjust the aliquot to give a residue spot within the range 0.005-0.1 µg. Spot standards and standard mixtures of 0.005, 0.01, 0.02, 0.05, 0.1 and 0.2 µg. Sample spots over 0.2 g are difficult to determine quantitatively and under 0.005 µg may be difficult to see. Spot all 6% Florisil eluates on one plate and 15% Florisil eluates on another plate.

For Phosphated pesticides: Adjust aliquots of sample and standards to give spots within the range 0.1-0.5 µg. Spot all Florisil eluates on the same plate. Ronnel, Ethion and Carbophenothion are not resolved. Spot standards of each of these separately. Spot Diazinon, Methyl Parathion and Malathion separately or as mixture.

The total volume of sample extract spotted should be less than 10 µl, if possible, and spotting should be done repeatedly with 1, 2 or 3 µl spotting pipettes. Use separate pipettes for each sample extract and standard. For best results, keep the size of the spots as small as possible.

Chromatography

Chlorinated pesticides: Place a filter paper liner and a metal trough in the tank. Presaturate the liner by pouring 75 ml of developing solvent into the bottom of the tank 30 minutes before developing the plate. Pre-saturation decreases the development time and improves uniformity of the R_f values. For plates spotted with 6% Florisil eluates, pour 50 ml of n-heptane into the trough. Place the

lower edge of the plate in the metal trough with the top of the plate leaning against the side of the tank. Place a glass plate on the tank and seal with masking tape. For plates spotted with the 15% Florisil eluates, use acetone and n-heptane (2 + 98) as the developing solvent.

Phosphated pesticides: Place a liner and a metal trough in the tank. Pour 50 ml of methylcyclohexane into the trough and 75 ml into the bottom of the tank. Quickly fill a dipping tank to within 4-5 cm from the top with immobile solvent. Invert the plate and dip with the uncoated side touching the back wall of the tank to prevent the front wall from scraping the adsorbent layer during the dipping operation. Dip the plate just to the spotting line, remove and immediately place in the metal trough with the top portion of the plate leaning against the side of the tank. Place a glass plate on the tank and seal with masking tape.

Visualization of Residue Spots

When the solvent front just reaches the pencil line 10 cm above the "spotting" line remove the plate and dry in a hood for 5 minutes.

For chlorinated pesticides: Support the plate on one side and spray fairly heavily with silver nitrate reagent, using lateral motions of the spray bottle. Spray until the plate appears translucent or soaked with reagent. Underspraying will result in poor sensitivity. After spraying, dry the plate in a hood for 15 min, then immediately place under a source of UV light for viewing. Expose the plate to UV light until the spot for the standard of lowest concentration appears. 5 ng of most chlorinated organic pesticides should be visible after about 15-20 min of exposure. Exposure times of 30 minutes generally will not harm the plates. For best results place the plates 8 cm from the bottom edge of the lamps. This distance should be adjusted based on experience.

For phosphated pesticides: Immediately spray the plate heavily and uniformly with dye solution, using lateral motions of the spray flask. The plate should be vivid blue after spraying. Overspray the plate lightly and uniformly with silver nitrate solution. At this point the plate should be bluish purple and spots should be discernible. After 2 minutes overspray the plate moderately and uniformly with citric acid solution. After spraying, thiophosphate pesticides should immediately appear as vivid blue or purple spots against a yellow background. The colour of the spots reaches maximum intensity about 5-10 min after spraying with citric acid. After about 10 min the background begins to change from yellow to greenish blue, masking the spots. At this point, respraying the plate with citric acid solution changes the background to yellow again and makes the spots stand out as well as or better than before. Evaluate the chromatogram 10 minutes after respraying. The blue spots fade completely and irreversibly 30-40 minutes after the time of the original citric acid spraying.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 29.003, 29.005-.006, 29.019-.027.

RESIDUE IDENTITY CONFIRMATION
(Using Paper Chromatography)

PRINCIPLE

See 5.1 of this Manual for a general discussion of paper chromatography technique.

APPARATUS

1. Developing tanks with glass tops.

2. Spotting pipettes.

3. UV viewing cabinet with short and long wave lamps.

4. Chromatographic paper, Whatman No. 1 or equivalent, 20x20cm.

REAGENTS

Aqueous System

1. Immobile solvent: dissolve 25 ml mineral oil (or corn, soya or cottonseed) in ethyl ether and dilute to 500 ml with ether.

2. Mobile solvents:

   a. 75% acetone in water

   b. 75% 2-methoxyethanol in water

   c. 75% methanol in water

   d. 40% pyridine in water

Non-aqueous System

1. Immobile solvents:

   a. Dilute 175 ml of N,N'-dimethylformamide (DMF) to 500 ml with ethyl ether.

   b. Dilute 50 ml of 2-phenoxyethanol to 500 ml with ethyl ether.

2. Mobile solvent - 2, 2, 4-trimethylpentane

Chromogenic Reagent

Place 1.7 g of silver nitrate in a 200 ml volumetric flask, dissolve in 5 ml of water, add 10 ml of 2-phenoxyethanol, and dilute to volume with acetone. (Add 1 small drop of 30% hydrogen peroxide solution to the flask just before diluting to mark.)

PROCEDURE

Preparation of Paper

With a hard pencil, rule an origin line 2.5 cm from the bottom edge and make a dot at 8-10 evenly spaced positions, with end dots 2.5 cm from the sides of the paper. With a pencil, mark a test number or other identification below each dot. Wash papers for aqueous systems several times with distilled water and dry before use. (Papers for non-aqueous systems need not be washed.)

Paper (especially washed paper) must be dry. (All air-dried papers should be further dried 30 minutes at 100-110°C before use.) If an appreciable amount of moisture is in the paper, it cannot absorb enough immobile solvent solution, and high R_f values result, as well as faint, indistinct chromatograms. Apparent air dryness is not adequate, drying in a forced-draft oven is necessary. Once dried, the paper may be kept in ordinary dry storage without adverse results. This moisture effect is most critical with non-aqueous systems.

(Note: once paper chromatography is started, spot, develop, spray with chromogenic agent and expose to UV light without delay; do not interrupt overnight.)

Sample Application

Take a portion of the cleaned sample extract equivalent to 2-5 µg of the pesticide residue to be confirmed. Evaporate under a gentle air stream at room temperature just to dryness. (Prolonged drying will result in loss of pesticide.) Wash down the sides of the tube with 0.5 ml of ether, evaporate and again wash down with 0.1-0.2 ml of ether. Evaporate, take up the residue (which is usually not visible) with 0.03-0.04 ml ether and transfer to one of the dots on the origin line of a chromatographic paper using a 1 µl pipette. Repeat until all the residue is placed on one spot. Let the spot dry after each application to restrict its size. Wash the tube again with 0.03-0.04 ml of ether and transfer to the same spot. Transfer the standard solutions of known pesticides to other dots on the same paper adjacent to the sample spots. Use additional pipettefuls to increase the amount of pesticide on a spot (one microlitre containing one microgram of standard). For identification place several compounds on separate dots on the same paper as the unknown.

Chromatography

After the samples and standards are spotted on the paper, put 50 ml of the mobile solvent in the tank trough. Fill the dipping tank with immobile solvent. Holding the paper by the bottom using a spring clip, immerse the paper top downwards into the immobile solvent just to the origin line, then immediately remove it. For an aqueous system, hang the paper to dry 2-3 minutes. While the paper is drying, clip a glass rod (which acts as a support for the paper in the tank) to the top of the paper opposite the origin line. When using the non-aqueous system, after dipping the paper in the immobile solvent, place it in the mobile solvent in the chromatography tank as quickly as possible without allowing time for drying. (As the ether evaporates it may condense moisture on to the paper and this interferes with the ability of pesticides to dissolve in the immobile solvent.) Excessive humidity and temperature tend to result in high R_f values and faint, indistinct chromatograms. Hang the paper in the tank so that the end near the origin dips about 1 cm into the trough filled with mobile solvent. Place a glass plate on top of the tank and seal with masking tape.

Visualization of Residue Spots

When the mobile solvent has risen up the paper to within 2.5 cm of the top (2 to 4 hours, depending on the solvent system used), unseal the tank, mark the solvent front and hang the paper up until it appears dry. Uniformly spray the dry paper with the chromogenic reagent (do not spray so heavily that it runs down the paper). Dry the paper until most of the solvent is removed and expose both sides to UV light until reduced silver spots are developed. (Darkening of the chromatogram background during storage may be largely prevented

by washing the finished chromatogram, after exposure to UV light, as follows: Suspend the paper from a glass rod with 3 or 4 clips and thoroughly play a gentle stream of distilled water on both sides of the sheet. Let the suspended papers hang until dry. Papers are very fragile when wet.)

It is advisable to evaluate chromatograms before washing them. Compare location, size and intensity of spots from unknowns with those from standatrds, identify and estimate pesticides semi-quantitatively. Always chromatograph knowns and unknowns on the same paper.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 29.004, 29.007, 29.028.

7.2 METALS

Analysis of metal residues in foods is a unique challenge to the laboratory. Organic food contaminant residues, such as pesticides, mycotoxins and others, are isolated for analysis using techniques usually involving partitioning or chromatography. Metal residues, however, cannot be isolated without destruction of the entire organic matrix. This often presents serious problems.

Another difficulty, common to all residues, is non-uniformity of the residue distribution through the food product. For example, the U.S. National Canners Association have shown the impossibility of accurately determining the level of lead in canned corned beef without analyzing the entire contents of the can. Sampling difficulties may be of far more importance than analytical errors in causing discrepancies in results. Contamination from grinding machinery may also invalidate analyses of some metals, particularly iron, tin and lead. Also, concentration of a contaminant may be distributed according to particle size of the food. Tea "dust" has been found to contain several times the amount of lead, iron, zinc and copper found in leaves retained on a 30 mesh sieve.

The destruction of the organic matrix of a food is accomplished by oxidation of the carbonaceous material while adding energy, usually in the form of heat. The two most common systems are dry ashing (air oxidation at high temperatures) and acid digestion (chemical oxidation at moderate temperatures). The ashing system for a specific metal will be given in the individual method for that metal. However, a discussion of dry ashing and acid digestion is given below.

Dry Ashing

Dry ashing is applicable to the determination of most common metals, usually with the exception of mercury, arsenic and tin. Substances amenable to this method must be charred slowly, and the carbon oxidised gently and completely. Loss of metals by volatilisation or by combination with the material of the container must be avoided by working at the lowest possible temperature. Particular care mut be exercised when large amounts of halogens are present in either covalent or ionic form. It has been reported that losses of certain metals (e.g. zinc, tin or antimony) occur when dry ashing is carried out in the presence of halides. Such losses can be minimized by ensuring that an alkaline ash remains.

Dry ashing usually requires little attention. Larger amounts of material can be dealt with more conveniently than by acid decomposition by repeated addition of fresh material to already ashed material and re-calcining. It is of particular advantage when the use of sulphuric acid is objectionable. For example, for the determination of lead in materials containing appreciable quantities of the alkaline earths, whose sulphates occlude lead sulphate. Dry ashing also avoids the use of large quantities of reagents and the potentially high blank values than can result from their use.

However, it is sometimes difficult to obtain complete extraction of the metal being determined from certain ignited residues, and excessive heating makes certain metallic compounds (e.g., those of tin) insoluble.

Certain flour products give a dark melt in which carbon particles are trapped and will not burn.

For foods having a relatively bulky ash straight ashing without an ash-aid is usually sufficient. The technique is as follows:

Weigh accurately a suitable quantity of the well mixed sample in a tared silica or platinum dish. Heat first by means of a gentle flame, such as that of an Argand burner, to volatilise as much as possible of the organic matter, then transfer the dish to a temperature-controlled muffle furnace, at a temperature

preferably not exceeding 420°C. Make sure the food emits only smoke during the flaming, and does not catch fire. This is especially true of fatty foods which will give copious smoke. Catching fire results in uncontrolled oxidation and possible loss of the metal residues. As an alternative to heating with an Argand burner, the sample may be placed in a cool muffle furnace (100°C to 150°C) and the temperature raised to about 420°C and left overnight. It is an advantage to draw a slight current of air over the sample during the early stages of the ignition to prevent carbon and tarry matter from being deposited inside the furnace.

Most substances can be ashed in a reasonable time at a temperature as low as 420°C if heated overnight, and such low temperatures are to be preferred. The time can be reduced by spreading the material in a layer over the dish. The temperature required will vary according to the material being ashed. In general the ash must not be sintered or fused, but with substances containing much ash, higher temperatures are required.

If it is suspected that all the carbon has not been removed, cool the ash, add a slight excess of dilute hydrochloric or diluted nitric acid (1 + 2), warm on a steam-bath, and note whether any colour is extracted or whether organic matter is still present. If so, evaporate the mixture to dryness on the steam-bath, and gently char the residue over a small flame until all the organic matter has been destroyed, or better, repeat the ignition at a higher temperature or for a longer period.

If a food has very little ash, or the metal residue is relatively volatile, then an ash-aid must be used. One of the common ash aids is a 50% magnesium nitrate solution. To prepare a solution free from metallic contaminants, adjust the pH of a 50% solution of magnesium nitrate to 9.5 with ammonium hydroxide, using thymol blue as indicator, and shake with successive portions of dithizone solution in chloroform until the dithizone layer remains green.

When using ash-aid, add about 2-5 ml of ash-aid solution to the sample. The solution and sample must be well mixed. The stirring rod may be wiped with a small piece of filter paper or cotton wool as long as hands are thoroughly clean and a similar quantity of paper or wool is included in the blank. Dry to remove moisture and ash as before. Materials such as milk and some confectionery can be charred without ignition by adding them a little at a time to a dish heated over a burner or hotplate. Oil and fat must be "smoked" away by heating at about 350°C. Covering the floor of the muffle with a sheet of refractory material such as silica causes most of the heating to take place by radiation from the walls and ceiling and facilitates this.

Acid Digestion

Dry ashing is preferably conducted in a fume hood. However, fume hoods are mandatory for acid digestions. The use of fume hoods constructed entirely of non-flammable materials is recommended, as there have been instances of wooden fume hoods taking fire after a long period of use with perchloric acid. On the other hand, many wooden-framed fume hoods have been in use for perchloric acid evaporation over a period of years without any untoward consequences. The hazard of using wooden fume hoods depends on the adequacy of ventilation, and the exposure of the wood to fumes from digestion vessels. In a known instance of conflagration, the use of perchloric acid was high and the fume hood was constructed of unprotected soft wood, which was subjected to considerable heat from an electric hot-plate.

It is suggested that, when a wooden-framed fume hood is used:

a. The wood should be teak.

b. The hood should have an efficient draught to prevent condensation of the acid vapours.

c. The fans should be left on for a while after digestions have been completed in order to clear residual fumes.

d. The woodwork should be washed down with water at frequent intervals and should be treated occasionally with liquid paraffin wax.

e. The wood should be examined occasionally. A splinter placed on a hot-plate will indicate whether or not the wood has become unduly inflammable.

f. The wood should be made non-absorbent by coating with an epoxy compound.

A satisfactory hood can be constructed from epoxy-resin-glass fibre laminates, but before any particular material is used a small fragment should be treated with hot perchloric acid and its resistant properties confirmed. Vitreous tiles provide the most suitable material for the bases of fume hoods. Dirt and dust must not be allowed to accumulate in fume hoods or fume ducts used for perchloric acid. A fume hood in which perchloric acid is used should not be connected to an ordinary chimney flue. Readily oxidisable cements, such as those made with litharge and glycerine, must not be used to seal cracks in fume hoods. Pastes of Alundum cement and sodium silicate or similar materials are satisfactory. Fume hoods incorporating water cooled baffles and scrubbing chambers and constructed of materials such as PVC are available and may be safely used with perchloric acid provided the manufacturer's recommendations are followed.

It is absolutely essential to use reagents and distilled water having very little or no metal content. One reason is that concentrated mineral acids are generally used in amounts several times that of the sample. Some of the more commonly used acids and reagents are now available in grades suitable for food analysis. They should not be transferred from the lead-free glass bottles in which they are supplied. However, even when these reagents are used, reagent blank determinations will be necessary. Blanks must be prepared with the same quantities of reagents as are used in the tests. Also, all glass or silica ware must be thoroughly cleaned with sulphuric and nitric acids and then thoroughly washed with distilled water immediately before use to ensure that it does not yield traces of metals under the conditions of test.

Acid digestion takes place most efficiently when there is some form of reflux action during the heating. This can be accomplished by use of a reflux condenser (as in the organic mercury analysis), but use of a Kjeldahl flask (with its long neck) is usually sufficient. The generation of hazardous fumes during the digestion is always a problem. If many digestions are being done at the same time, the fume hood may not be able to handle the fumes. One method of fume control involves constructing a fume collector as follows: cut a 100 mm diameter PVC pipe to fit horizontally in an available fume hood. Cap both ends with PVC caps and epoxy sealer. Drill a series of holes along the pipe (about 140 mm between centres) which will loosely accommodate the neck of a Kjeldahl flask. Drill a single hole at one end to allow a brass (not copper) or PVC tube of size to be attached with epoxy and connected to vacuum tubing. The vacuum tubing would go to a water aspirator attached to a faucet in a sink. With the faucet on, a sufficient vacuum is formed to draw the fumes out and dispose of them by mixing with water in the aspirator.

Digestion of organic matter is usually done using sulphuric acid as the solutional material. Nitric acid is the most common oxidant, with perchloric and hydrogen peroxide used for difficult foods. Metal or metal oxide catalysts may also be used to enhance the oxidation. A caution should be noted if the sample contains large amounts of alkaline earth metals. Their insoluble sulphates will often absorb or occlude trace metals such as lead. In such events, sulphuric acid cannot be used and nitric and perchloric acids are necessary. Extreme care must be taken when perchloric acid is used, to avoid explosions or overly rapid oxidations.

When sampling beverages, it is often best to take a portion of the beverage equivalent to about 5 g of solids. If in doubt, this can be determined by evaporation of a known amount of the beverage. Any sample (such as a beverage) containing a large proportion of water, must be boiled down to a smaller bulk (after adding nitric acid), before adding the sulphuric acid and additional nitric.

When a less reactive food is to be digested, add only nitric acid first and heat until the reaction subsides, then add the sulphuric acid and continue digestion.

If a food is extremely reactive to nitric, add the acid in dilute (1 + 2) form and warm gently until the initial vigorous reaction is over. Often, at this point a spongy, tarry cake is formed. Cool the mixture, pour off the acid into a beaker, and wash the tarry residue with a small amount of distilled water (three or four 1-ml portions), adding the washings to the acid liquor in the beaker. Add 8 ml of sulphuric acid to the tarry residue, agitate to disperse the cake, and introduce nitric acid, drop by drop, with warming if necessary, until vigorous oxidation ceases. Return the original acid liquor to the flask, and boil until the solution just begins to darken, then continue the digestion. A blank must be prepared. When several samples are being digested, they and the blank must eventually receive the same amounts of acid so that any metallic impurities in the reagents cancel out.

Remember that for an effective digestion a small, but not excessive, amount of free nitric acid must be present throughout the digestion. Continue adding small amounts of nitric as necessary until the solution fails to darken on prolonged heating to fuming (5 to 10 minutes). The criterion of completion of oxidation is that the final solution is fuming when hot and colourless when colder, but if much iron is present the solution will be pale yellow in colour, frequently with a granular precipitate soluble on dilution. Allow to cool somewhat, dilute the solution with 10 ml of distilled water (this should give a colourless solution, or a faintly yellow one if iron is present), and boil gently to fuming. Allow the solution to cool again, add a further 5 ml of distilled water, and boil gently to fuming. Finally, cool, and dilute the solution with 5 ml of distilled water. The digestion is now complete.

When perchloric acid is used, it can considerably reduce the amount of nitric acid required and complete the oxidation in a shorter time, provided that the presence of chloride is not detrimental to the procedure for determination of the metals. It is most important to read the safety precautions relating to perchloric acid before boiling it. Some important properties and uses of perchloric acid are:

1. Perchloric acid is completely stable under ordinary storage conditions in concentrations of less than 85%. The concentrations normally supplied are 60% and 72%.

2. The azeotropic mixture with water contains 72.5% of perchloric acid and boils at 203°C at 760 mm pressure, so that the evaporation of an aqueous solution of perchloric acid can never produce an acid of dangerously high concentration. If, however, metallic salts are present, the mixture should not be evaporated to dryness over an open flame.

3. Perchloric acid vapour and inflammable gases form violently explosive mixtures, and care should be taken to avoid their formation.

4. Hot 60 to 72% perchloric acid is a powerful oxidising agent and oxidises all forms of organic matter, but it loses its oxidising properties entirely when cooled and diluted with water. The constitution and properties of any material must be taken into consideration before treatment with perchloric acid, whether the material be vegetable or animal matter or a pure chemical. Generally, the speed of the reaction can be easily controlled, but

samples containing alcohol, glycerol or other substances that form esters should not be heated with perchloric acid or perchloric acid mixtures, except under previously well tried conditions.

5. When possible, nitric acid should be present during an oxidation by perchloric acid. It should be added before the perchloric acid and definitely before evaporation to fumes. The effect of nitric acid is to moderate the reaction by oxidising the more reactive components at lower temperatures. It should be remembered, however, that heterocyclic substances containing nitrogen are as a rule not readily oxidised by nitric acid, and the possibility of delayed reactions should not be overlooked.

6. Some inorganic materials, such as hypophosphites and tervalent antimony compounds, also tend to form explosive mixtures with perchloric acid when hot. A large excess of nitric acid should always be present when oxidising inorganic salts.

7. Perchloric acid should not normally be used for oxidising organic materials with which it is highly reactive but immiscible, as the reaction is localised in the zone of contact. It should therefore not be used for oxidising materials containing much fat until any excess of fat has been removed, since local over-heating cannot be controlled and the temperature may rise dangerously. The oxidation of other substances such as sulphur is, however, slower and readily controlled.

8. When perchloric acid is used as a dehydrating agent as, for example, in the determination of silica, with subsequent filtration, the residue must be thoroughly washed with dilute hydrochloric acid before the filter paper is ignited.

9. The use of a face shield is strongly advised when perchloric acid is being used in laboratory procedures, as well as protective clothing.

10. Perchloric acid is supplied, and should be stored, in glass-stoppered bottles. To prevent the possibility of hazard in the event of breakage, perchloric acid should be kept apart from organic chemicals and reducing substances, especially alcohol, glycerol and hypophosphites. Bottles of perchloric acid should not be kept on wooden shelves or benches, since acid trapped in the stopper joints may spill on to the benches and these may at some future time become heated and ignite. The bottles should stand in glass or porcelain dishes or on ceramic or other non-flammable and non-absorbent benches, preferably in such a position that the acid can easily be washed away in the event of breakage or spillage.

11. Any acid spilled should be diluted with water. Swabs used to wipe it up should be, if possible, of wool waste or some other non-flammable material and not of cellulose.

12. Perchloric acid that has become discoloured should be diluted with water and washed away.

When either perchloric or 30% hydrogen peroxide are used, the food is normally digested in great part using nitric/sulphuric acids before addition of the more vigorous oxidizing agents. As example, perchloric acid or hydrogen peroxide in small amounts (0.5 ml) can be added after the sample has been digested to a pale yellow solution using nitric. The digestion is then continued until the solution is colourless and white fumes are obtained. The white fumes are sulphur trioxide and indicate that all nitric (or other) oxidation products are gone. Cooling and adding 10 ml water and digesting to white fumes again (twice) will eliminate all nitrogen oxides.

In the event that sulphuric acid may not be used, then nitric acid must be added to the food (about 25 ml for each 2 g), and the mixture boiled for about 30 min. Cool and add 15 ml 60% perchloric. Boil gently until fumes appear. Do not boil to dryness as this can be dangerous.

The advantages of using concentrated hydrogen peroxide are the avoidance of nitric acid fumes and the low reagent blank values. Oxidation is catalyzed by various metal compounds, especially their oxides and hydroxides and by precious metals. It is accelerated by alkaline conditions.

Hydrogen peroxide should be stored and used under such conditions that, through spillage, it does not come into contact with combustible materials. Spilled peroxide solution should be washed away at once with water, or wiped up with dilute ammonia solution, as higher concentrations are produced on evaporation, and spontaneous ignition of inflammable materials can result. Rubber or plastic gloves should be worn when handling the reagent. In contact with the skin it immediately produces "white burns". Any burns must be washed immediately with water or dilute potassium permanganate solution, otherwise they will become painful and may cause blistering. Eye protection is essential, and oxidation procedures should be carried out behind a safety screen in a fume hood.

For the purposes of oxidation, 50% or 30% hydrogen peroxide must always be used in conjunction with a sufficient amount of sulphuric acid. Explosions have been produced deliberately by evaporating large volumes (about 50 ml) of peroxide with small volumes (less than 5 ml) of sulphuric acid. Though noisy, the explosions did not produce mechanical fracture of the glassware involved.

The following table gives some volumes of 50% peroxide and sulphuric acid required for some selected food products:

	Sulphuric acid fuming when hydrogen peroxide added			**Sulphuric acid not fuming when hydrogen peroxide added**		
Food	H_2O_2 ml	H_2SO_4 ml	Minutes for complete digestion	H_2O_2 ml	H_2SO_4 ml	Minutes for complete digestion
Soya Bean Oil	15 to 20	10	15 to 20	10 to 15	5	20
Cabbage	5 to 10	10	5 to 10	5	5	10
Cheese	15 to 20	10	10	10 to 15	5	10 to 15
Meat	10 to 15	10	10	10 to 15	5	10

LEAD
(Colorimetric Method)

PRINCIPLE

Lead may occur naturally in some foods in small amounts, but is usually present as a contaminant. Lead can enter food from environment or from contact with processing equipment or storage containers having lead-containing materials (these could be solders, paints, ceramic glazes, etc.) Dust in the laboratory atmosphere or settled on surfaces may contain lead, especially if the laboratory is near a road carrying a lot of vehicular traffic. The determination should be carried out in diffuse light as bright sunlight tends to decompose dithizone and dithizonates.

A choice must be made between ashing and acid digestion. Both methods work satisfactorily on most foods as long as certain ground rules are followed. If the ash content is very low and the sample is to be ashed, ash-aid should be added. This is because lead is strongly retained on silicates, whether those in the sample or the walls of the silica or porcelain dish used. Aluminium and calcium oxides act as a matrix through which the lead can more easily remain dispersed. Foods containing relatively high amounts of silicates should be digested with acid. Samples high in calcium should not be digested with sulphuric acid as the calcium sulphates occlude lead. High chloride levels may lead to loss of lead by volatilisation. Although sodium chloride cannot be responsible for loss in this way, ammonium chloride can. (The chlorides of ammonium, magnesium and calcium all cause loss of zinc on ashing.) Samples high in chloride may be digested in acid. Alternatively, add dilute sulphuric and evaporate slowly so that chloride is lost as hydrochloric acid at around 100°C and then ash in the normal way.

This method involves destruction of organic matter by dry ashing and separation of the lead from interfering substances. The lead is extracted into chloroform as the red-coloured dithizonate, and is determined spectrophotometrically.

APPARATUS

1. Ashing dishes, porcelain or silica.

2. Muffle furnace.

3. Separatory funnels.

4. Volumetric flasks.

5. Spectrophotometer.

REAGENTS

1. Ammonia-citrate - dissolve 400 g citric acid in ca 800 ml water. Add NH_4OH until alkaline to phenol red (pH 8.4). Dilute to 1 litre. Purify by extracting with dithizone solution.

2. Hydroxylamine hydrochloride solution - dissolve 20 g $NH_2OH.HCl$ in water to make 65 ml. Add a few drops of meta-cresol purple indicator. Add NH_4OH until the solution turns yellow (pH 2.8). Add an excess of 1% diethyldithiocarbamate solution. Extract with $CHCl_3$ until the yellow colour is gone. Add HCl until the solution is pink (pH 1.2). Make to a total volume of 100 ml with water.

3. Potassium cyanide solution - dissolve 100 g KCN in water and make to 1 litre. Add 10 g MgO and boil gently for 30 min. Cool, filter using vacuum and dilute to 1 litre with water. NOTE: Cyanide must be handled with great care, expecially by those who are not sensitive to the odor.

4. Dithizone (diphenylthiocarbazone) solution - Extract (in a separatory funnel) 1 litre of $CHCl_3$ with 100 ml water containing 0.5 g $NH_2OH.HCl$ and made alkaline to phenol red using NH_4OH. Drain and collect $CHCl_3$ and immediately add 5 ml ethanol (as a stabilizer). Dissolve 30 mg dithizone in this $CHCl_3$. Keep in refrigerator. Extract with 1% HNO_3 just before use.

5. Ammonia - cyanide solution - dissolve 10 g KCN in one litre NH_4OH. (See 'NOTE' above.)

6. pH 3.4 buffer solution - dilute 9.1 ml HNO_3 to 500 ml with water and adjust pH to 3.4 with NH_4OH. Add 25 ml 0.4 N KH phthalate and 5 ml of 0.2N HCl. Make to 1 litre with water. Check with pH meter before using. Adjust pH to 3.4 with NH_4OH, if necessary.

7. Lead standard solution - dissolve 3.197 g lead nitrate (dried at 100°C) in 1% HNO_3 and dilute to 1 litre with 1% HNO_3. This is the stock solution (2 mg Pb/ml). Prepare the working standard solution (2 µg Pb/ml) by diluting 1.0 ml of the stock to 1 litre using pH 3.4 buffer solution.

PROCEDURE

Weigh 20-40 g (to nearest 0.1 g) sample into ashing dish. Dry samples several hours or overnight in 120°C forced-draft oven. (Sample must be absolutely dry to prevent spattering in furnace.) Place samples in furnace set at 250°C. Slowly raise temperature (50° increments) to 350°C and hold at this temperature until smoking ceases. Increase temperature to 500°C in about 75°C increments (sample must not ignite). Ash 16 hr (overnight) at 500°C. Remove from furnace and allow to cool. Ash should be white and essentially C-free. If ash still contains excess carbon particles (i.e. ash is grey rather than white), then wet with minimum amount water followed by dropwise addition of nitric acid (0.5 - 3 ml). Dry on a hotplate. Transfer to the furance at 250°C, slowly increase the temperature to 500°C and continue heating 1-2 hr. Repeat the acid treatment and ashing if necessary to obtain C-free residue.

Dissolve residue in 5 ml 1N nitric acid warming on steam bath or hotplate 2-3 min to aid solution. Filter, if necessary, into 100 ml volumetric flask. Repeat with two 5 ml portions 1N acid, filter and add washings to original filtrate. Dilute to mark with 1N acid. Prepare duplicate reagent blanks for standards and samples, including any additional water and acid, if used for sample ashing. (Note: do not "ash" nitric acid in furnace, since lead contaminant will be lost. Dry nitric in ashing dish on steam bath or hot plate, and then proceed.)

Shake the flask thoroughly, then pipette 50 ml to a separatory funnel containing 15 ml of the ammonia-citrate solution. Make alkaline to phenol red (pH 8.4) using ammonia, then cool. Add 5 ml KCN solution, 1 ml $NH_2OH.HCl$ solution and 5 ml dithizone solution. Extract by shaking.

If dithizone layer remains green, there is little or no lead present. Drain the dithizone in a second separatory funnel and continue from the water wash step below.

If the dithizone is red or reddish-green, drain into a second funnel and re-extract with 5 ml portions of fresh dithizone until one remains green. Combine all extracts in the same separatory funnel. Wash the combined dithizone extracts with 50 ml water. Drain the dithizone into a third separatory funnel. Discard the water wash.

Add 50 ml pH 3.4 buffer solution to the dithizone in the third funnel, and extract by shaking. (This will strip the lead from the dithizone into the buffer solution. The dithizone should turn green. If it does not then bismuth is present - see below.)

Discard the dithizone layer. (If bismuth is present, extract the buffer solution with two 5 ml portions of fresh dithizone and discard them.)

Add 20 ml of the ammonia-cyanide solution to the buffer. Pipette 10.0 ml fresh dithizone solution into the funnel with the buffer, and shake 1 min. (Note - release pressure through the stopper, rather than the stopcock.) Place a small plug of cotton in the stem of the separatory funnel. Drain the dithizone through the cotton, discarding the first 1-2 ml, into a 1 cm cuvette.

Determine the absorbance at 510 nm vs fresh dithizone solution as the reference. Make a procedural blank determination by running an analysis using all reagents, but without the sample.

Prepare a standard curve as follows: Pipette 0.0, 5.0, 10.0, 15.0 and 25.0 ml of the lead working standard (2 µg Pb/ml) into five separatory funnels. Add pH 3.4 buffer to each funnel to make a total volume of 50 ml in each. Next add 20 ml of the ammonia-cyanide solution to each funnel and continue the analysis from "...Pipette 10.0 ml fresh dithizone...". Record the absorbances and prepare a standard curve plotting absorbance vs lead concentration.

CALCULATION

$$\text{Lead ppm} = \frac{A}{S} \times \frac{100}{50}$$

where:

A = µg of lead corresponding to the sample absorbance (taken from the standard curve)

S = Weight of sample in g

INTERPRETATION

Lead recovery, when added to a lead-free food, should be at least 80% in the 10-40 µg range.

Any substances remaining undissolved, either after digestion, or on making the digest alkaline, are likely to cause low results, due to occlusion of lead in the precipitate. Therefore, if a sample contains more calcium or magnesium phosphates than can be held in solution by ammonium citrate (e.g. cacao products, tea, sardines) the lead must be separated from the phosphate before completing the colorimetric determination.

Addition of higher amounts of citrate than those recommended may lead to incomplete extraction of the lead. Tin in excess of about 150 mg/kg may result in a milky suspension of SnO_2 in the ash solution or precipitate when the digest is made alkaline. In either case the interference must be removed.

Removal of Excess Phosphate (Society of Analytical Chemistry Method):

To the solution prepared from the ash or digest, add 2 drops of methyl red indicator. Make just alkaline and add a further 10 ml. Warm to 50°C and add 2 ml of 20% sodium iodide solution. Reduce any liberated iodine with 2 ml of 1.25% sodium metabisulphite solution. (Iodine oxidises dithizone). Cool and transfer to a separating funnel and adjust the volume to 50 to 75 ml (to bring the acid concentration to N with respect of hydrochloric acid). Add 10 ml of

1% diethylammonium diethyl carbodithioate reagent in chloroform by pipette and shake the funnel vigorously for 30 seconds. Allow the layers to separate and transfer the chloroform layer to a 100 ml flask. Wash the aqueous layer twice with small amounts of chloroform without mixing and add these washings to the flask. Repeat the extraction with 10 ml of carbodithioate reagent and add the second extract to the main extract. Discard the aqueous layer. To the combined extracts add 2.0 ml of diluted sulphuric acid and evaporate the chloroform. Add 0.5 ml of perchloric acid to the residual solution and heat until fumes are evolved and the fuming solution is clear and colorless. Cool the solution, add 10 ml of water and 5 ml of 5 N hydrochloric acid, boil for 1 minute, cool, and then transfer to a 100 ml volumetric flask using 1 N nitric acid. Dilute to volume using 1 N nitric. Pipette 50 ml into a separatory funnel containing 15 ml of the ammonia-citrate solution and continue the analysis from there.

Removal of Large Amounts of Tin (AOAC Method):

After an almost C-free ash is obtained, add 15-20 ml 40% redistilled hydrobromic acid. If nitrates were used as ash aids, cover the crucible with a watch glass and heat on a steam bath until bromine evolution diminishes, then rinse the watch glass with water and bring to the boil to complete expulsion of bromine. (This process destroys undecomposed nitrates). Add more HBr if necessary to dissolve ash and examine the solutions for clearness. If there is an insoluble residue of SnO_2, add 50-100 mg pure Tin to the simmering HBr solution of the ash and let it dissolve. (Metallic tin is the best agent to bring ignited SnO_2 into solution. To be effective, the ash solution must be in reduced state. Fe_2O_3 sometimes becomes "noble" during ashing and dissolves with difficulty, but treatment with metallic tin also brings it into solution. Treatment with tin is necessary only with contents of badly corroded cans.)

When the solution of ash is free from milkiness due to SnO_2, add 20 ml 60% perchloric acid, oxidize the mixture with few ml HBr-Br_2 mixture, and then add additionally 15 ml of the reagent a little at a time, while the solution is evaporated to incipient fumes (ca 150°C) on a hot plate. Repeat with an additional 10 ml portion of the HBr-Br_2 mixture if 100 mg Sn was used to dissolve the ash. (Hot perchloric helps keep ash salts in solution and with Br_2 holds Sn as volatile $SnBr_4$). When HBr and Br_2 are completely volatilized, cool, and take up with hot water (200 ml may be necessary if much chlorate is present). Filter off any small amounts of dehydrated SiO_2, extract residue twice with 5 ml hot 20% HCl-20% citric acid reagent and hot water and isolate lead by dithizone extraction.

REFERENCES

Manual for Food Canners and Processors, Vol. 2, U.S. National Canners Association, 1976.

Official Methods of Analysis of the AOAC, 1984, 25.126.

LEAD AND CADMIUM
(Atomic Absorption Method)

PRINCIPLE

Lead and cadmium are extracted from a solution of the sample ash. Standards are treated in the same way and both sample and standard extracts are aspirated in the flame of an atomic absorption spectrophotometer.

APPARATUS

(Before use, all items of glassware and silica dishes should be immersed in 5% hydrochloric acid for several hours, and then rinsed with double-distilled water.)

1. Lipped silica dishes, volume about 30 ml.

2. Graduated 5 ml and 1 ml pipettes.

3. 25-ml volumetric flasks with plastic stoppers.

4. Atomic absorption spectrophotometer, with recorder. (The operating conditions for lead and cadmium, using an Atomspek H1170, (Hilger and Watts) are:

	Lead	Cadmium
Lamp current (mA)	6	5
Wavelength (nm)	217.0	228.9
Slit width (m)	100	45
Burner height (mm)	9	9
Acetylene (litre min^{-1} at 5 psig)	0.75	0.75
Air (litres min^{-1} at 30 psig)	2	2
Scale expansion	1.0	0

REAGENTS

1. Water: Double-distilled, using silica-sheathed elements.

2. Nitric acid, low-in-lead quality.

3. Hydrochloric acid, low-in-lead quality.

4. Diethylammonium diethylcarbodithioate (DDCD): A 1% w/v solution in methyl isobutyl ketone. The solution may be kept for several weeks without deterioration.

5. Methyl isobutyl ketone (MIBK) solvent saturated with water.

6. Ascorbic acid freshly prepared, 10% aqueous solution.

7. Standard solution of lead and cadmium: A 2% w/v solution of hydrochloric acid containing 10 ppm of lead and 2 ppm of cadmium, prepared from appropriate salts.

PROCEDURE

Dry ash 10g of homogenised sample at 450-500°C in a silica dish until the ash is grey or white. Moisten with diluted nitric acid/water (1 + 9) and briefly re-ash if necessary. Treat the ash with 5 ml of water followed by 5 ml of hydrochloric acid and evaporate to dryness on a steambath. Add 1.0 ml of hydrochloric acid and 3-5 ml of water, stirring with a glass rod, filter into a 25-ml volumetric flask and make up to the mark with dish and rod rinsings. Prepare aqueous

standards in 25-ml calibrated flasks using 0, 0.10, 0.20, 0.50 and 1.00 ml of the standard lead/cadmium solution. To each flask add 1.0 ml of hydrochloric acid and make up to the mark with water.

To the blank, standards and samples add 0.5 ml of ascorbic acid solution (to prevent stannous interference) and invert to mix the contents. Then, without delay, add 1.50 ml of DDCD solution, stopper and shake the flasks for 30 seconds. When the phases have separated, tap the flasks on the bench to remove solvent globules caught on the sides of the flasks and aspirate the organic layers into the spectrophotometer first for cadmium and then for lead. Aspirate MIBK solvent before and after each sample or standard in order to establish the baseline. Construct calibration graphs of the responses to the standards.

CALCULATIONS

Compare the sample absorbance to the appropriate standards (after subtracting the blank) and calculate the amounts of lead and cadmium present.

INTERPRETATION

Extraction of lead and cadmium from 0.5 N HCl by DDCD in MIBK is a convenient alternative to the colorimetric procedure. If an atomic absorption spectrophotometer is not available, the lead and cadmium may be extracted with DDCD/MIBK, using a somewhat larger volume and repeating the extraction with fresh DDCD to ensure complete extraction. The organic phase must be evaporated, digested in the minimum amount of acid, and the determination completed with dithizone. Calcium, magnesium and phosphate are unlikely to cause difficulties of precipitation in 0.5 N hydrochloric acid.

Of the elements likely to be present in food digests, only iron, copper and zinc could interfere in this determination. These elements are usually masked by forming their cyanide complexes at pH 8.5. In this method it has been found necessary to eliminate completely the interference from iron by reducing any ferric to ferrous with ascorbic acid prior to the extraction step.

Experiments have indicated that the lead-DDCD complex is stable for several hours, while that of cadmium is subject to slight decomposition (judged by a decrease of 10 to 20 percent in the instrument response). For this reason cadmium is best determined first and the lead determination completed within three hours of extraction.

REFERENCES

Roschnik, R.K., 1973. Analyst 98, 596-604.

Official Methods of Analysis of the AOAC, 1984, 25.061-.065.

ARSENIC
(Colorimetric Method)

PRINCIPLE

The sample is digested using acid. Dry ashing should not be used because of the volatility of arsenic. The arsenic is converted to As_2O_3 during the digestion process. This is then reduced to arsine (AsH_3) by the hydrogen generated by acid and zinc. The arsine is reacted with a solution of silver diethyldithiocarbamate to form a red complex. This is measured using a spectrophotometer at 522 mm.

APPARATUS

1. Acid digestion flasks.
2. Volumetric flask, 250 ml.
3. Spectrophotometer.
4. Generator apparatus - as shown.

Use 50-60 ml wide-mouth bottles of uniform capacity and design as generators and fit each by means of a perforated stopper with a glass tube which broadens into a section 10 x 60 -70 mm. Place a small wad of glass wool in the constricted bottom end of the tube and add 3.5-4 g sand, taking care to have same amount in each tube. Moisten the sand with 10% lead acetate solution and remove excess by light suction. Clean the sand when necessary by treatment (do not remove sand from tube) with nitric followed by water rinse and suction. Treat with lead acetate solution. If sand has dried through disuse, clean and remoisten as directed. Connect the tube by means of a rubber stopper, glass tube, and rubber sleeve to a bent capillary tube (7 mm outside diameter, 2 mm inside diameter) tapered at the lower end such that it slides into the rubber sleeve to form a gas-tight seal and can also be placed in the neck of a 25 ml volumetric flask. The upper end of the tube expands into a trap of about 10 ml capacity tapered by a B19 joint, about a quarter full with small glass beads. Clean the trap between determinations without removing beads by flushing with water followed by nitric acid, soaking for 30 minutes and until the nitric acid becomes colourless. Remove all traces of acid with water, rinse with acetone and dry with a current of air by applying suction to the tip of the trap.

REAGENTS

1. Nitric acid, concentrated.

2. Sulphuric acid, concentrated.

3. Ammonium oxalate solution, saturated.

4. Sea sand - clean sand by treating with concentrated nitric acid and then rinse with distilled water to remove all traces of acid (at least 5 washings).

5. Lead acetate solution - 10% $Pb(OAc)_2.3H_2O$ in water.

6. Hydrochloric acid, concentrated.

7. Potassium iodide solution - 15% KI in water - keep in the dark and discard when solution turns yellow.

8. Stannous chloride solution - 15% $SnCl_2.2H_2O$ in HCl.

9. Silver diethylcarbodithioate reagent - 0.5 g dissolved in pyridine and made to 100 ml with pyridine. Store in an amber bottle. (The reagent is stable for several months at room temperature.)

10. Zinc, granulated or small pellets.

11. Arsenious oxide (As_2O_3) standard solution - dissolve 1.32 g As_2O_3 (equivalent to 1.00 g As) in 25 ml 20% NaOH solution. Dilute to 1 litre with water. This is the stock solution (1 mg/ml). The working solution is made by diluting 1 ml of the stock solution to 1 litre with water (1 µg/ml).

PROCEDURE

Weigh 25 g sample into digestion flask. Add 40 ml each of nitric and sulphuric acids. Digest with heat. Add a small amount of nitric whenever the digest turns brown or darkens. Continue digestion until organic matter is gone. The final solution should be nearly colorless. Stop digestion when white SO_3 fumes appear.

Cool and add 75 ml water. Next add 25 ml saturated ammonium oxalate (to complete removal of nitrogen oxides). Heat to evaporate the water and continue heating until white SO_3 fumes are formed again. Cool and transfer to a 250 ml volumetric with water. Make to volume with water.

Pipette 5 ml to a generator bottle and add 30 ml water. Add (while swirling) 5 ml HCl, 2 ml KI solution and 8 drops of the $SnCl_2$ solution. Let stand 15 min. Add 4.0 ml of the silver colour reagent to the trap of the generator apparatus. Add 4 g zinc to the bottle and quickly attach the scrubber tube and trap assembly,. Let react for 30 min. Transfer the silver reagent from the trap to a cuvette and determine the absorbance at 522 nm vs water. (Note - cuvette should be capped.)

Prepare a standard curve as follows: Pipet 0.0, 1.0, 3.0, 6.0, 10.0 and 15.0 ml of the working standard (1 µg As/ml) into six generator bottles. Make each to a total volume of 35 ml with water. Proceed as above. Prepare a standard curve, plotting absorbance vs µg As.

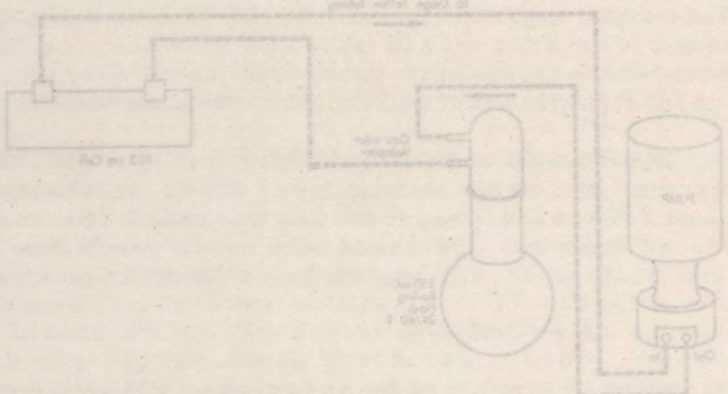
CALCULATIONS

Read µg As corresponding to sample absorbance, from standard curve.

$$\text{Arsenic ppm} = \frac{\mu g \text{ As} \times 250}{g \text{ sample} \times 5}$$

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 25.041-.044.



MERCURY
(Organic Residues)

PRINCIPLE

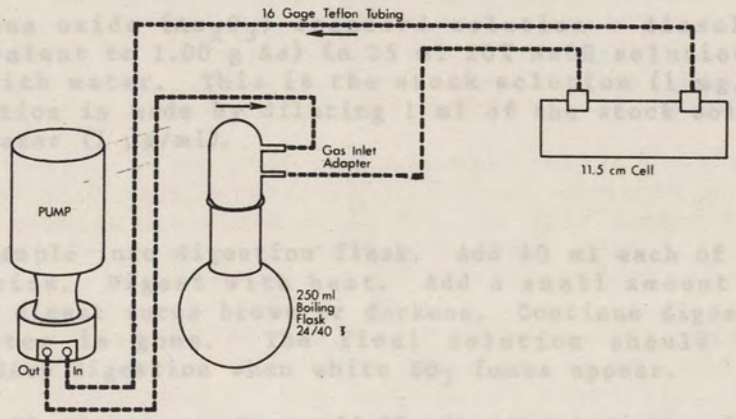
Organic mercury is commonly found in the flesh of fish. It is passed up the food chain from prey to predator and is cumulatively stored by each host. Therefore, the larger predator fish at the top of the food chain have the most likelihood of high residues of organic mercury. This method acid digests the fish using heat, oxidizing conditions and a catalyst in order to break the mercury from its organic bond. The resultant mercuric ion is reduced to elemental mercury with stannous chloride. The mercury is then aerated from the solution as a vapor into a chamber through which light at the mercury wavelength is passing. The resultant light absorbance is measured.

APPARATUS

1. Digestion flask and reflux condenser.

2. Volumetric flasks and pipettes.

3. Mercury Analyzer, Coleman or equivalent (see diagram below). Many atomic absorption spectrophotometers have a cold vapour accessory for mercury analysis.



REAGENTS

1. Vanadium pentoxide (V_2O_5) powder.

2. Sulphuric - Nitric acid mixture (1 + 1).

3. Hydrogen peroxide (H_2O_2), 30%.

4. Diluting solution - mix 58 ml HNO_3 and 67 ml H_2SO_4 with ca 500 ml water. Dilute to 1 litre.

5. Reducing solution - Mix 50 ml H_2SO_4 with 300 ml water. Cool and dissolve 15 g NaCl, 15 g hydroxylamine HCl and 25 g $SnCL_2$. Dilute to 500 ml.

PROCEDURE

Weigh 5 g prepared fish into digestion flask. Add some boiling chips, 10-20 mg V_2O_5 and 20 ml H_2SO_4-HNO_3 acid mixture. Connect flask to condenser and heat to produce a gentle boil in about 5 min. Continue heating with a strong boil for about 20 min. The solution should be clear. Ignore any fat globules floating on top.

Remove heat and wash down condenser with ca 15 ml water. Add 2 drops H_2O_2 and wash into flask with another 15 ml water. Cool flask to room temperature and disconnect condenser. Transfer digest into 100 ml volumetric flask using water to wash. Make to volume with water.

Pipette 20 ml into aeration bottle and add 80 ml diluting solution, Add 20 ml reducing solution and immediately aerate. Read directly from Analyzer meter. (Analyzer should be calibrated at 0.5 µg Hg).

CALCULATION

Read the micrograms mercury directly from the Analyzer Meter. This is also the ppm as 1 g sample equivalent is in the final solution.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 25.134-.137.

MERCURY
(Inorganic Residues)

PRINCIPLE

The sample is digested in nitric and sulphuric acids under reflux. The mercury is isolated by dithizone extraction, copper is removed and the colour of the mercury dithizonate compared with standards.

APPARATUS

1. Reflux apparatus (see Diagram). "A" is about 200 ml capacity and about 50 mm outside diameter. Friedricks condenser or equivalent should be used. "B" has a capacity of about 75 ml.

2. Spectrophotometer.

REAGENTS

1. Mercury standard: (Add 8 ml hydrochloric acid per litre to all standards before diluting to volume.) Dissolve 0.1354g mercuric chloride in 100 ml water (equivalent to 1gHg/L). Dilute to a working standard of 2 µg/ml.

2. Chloroform: Distil on a hot waterbath, collecting the distillate in 10 ml absolute alcohol per litre of distillate. Shake the receiver intermittently during distillation.

3. Dithizone: 100 mg/L in redistilled chloroform. Dilute as required.

4. Sodium thiosulphate solution: 1.5%. Prepare on day of use.

5. Sodium hypochlorite solution: Preferably with 5% available chlorine content. Check by titration. Store in the refrigerator when not in use. Certain preparations intended for household use contain traces of mercury. If these are used, check the blank value and discard the reagent if 0.1 µg/ml Hg or more is present.

6. Dilute acetic acid, 30% V/V.

7. Hydroxylamine hydrochloride solution: 20% w/v. Extract with dilute dithizone until the chloroform layer remains green, remove the excess dithizone with chloroform and filter.

PROCEDURE

Weigh an amount of sample containing less than 10 g of dry matter. Digestion must be almost complete, otherwise residual organic matter may combine with mercury and prevent or hinder extraction with dithizone. Oxidizing materials in the digest must also be destroyed otherwise the dithizone is decomposed and mercury is not quantitatively extracted. Careful heating of the digest during sample preparation is necessary because of the volatility of mercury compounds. The acidity of the final digest (after partial neutralization with ammonia) before extraction should be about 1 N and not more than 1.2 N. Do not use silicone grease in the stopcocks.

Fresh Fruits and Vegetables and Beverages

Place the weighed sample in the digestion flask with 6 glass beads, connect the assembly and add, through the dropping funnel, 20 ml nitric acid. Pass water through the condenser as rapidly as

practicable. With the stopcock to the Soxhlet portion open and that of the separator closed, heat the flask gently. The original reaction must not proceed with such violence that evolved NO_2 carries digest vapours through the condenser and causes loss of mercury. After the initial reaction is complete, apply heat so that the digest just refluxes. If the mixture darkens, add nitric acid dropwise through the funnel as needed. Note the total volume of nitric acid used so that a blank can be prepared identically. Continue refluxing half an hour or until the digest does not change consistency, then cool.

Slowly add 20 ml of cold nitric-sulphuric acids mixture (1+1) (or 10 ml if the dry weight of sample is less than 5g). Heat with a small flame, adding nitric acid dropwise as needed to dispel darkening of the digest. Continue heating until fibrous material (fruit skin, cellulose, etc.) is apparently digested. Turn the stopcock of the Soxhlet unit so that water and acid that distils is trapped and continue heating. Let the digest become dark brown (not black) before adding further increments of nitric acid. Fats and waxes cannot be totally digested by the hot acids under reflux, so no attempt should be made to effect complete digestion. When all except fat and wax is in solution, let the digest cool, and cautiously drain the water and acids into the main digest. Cool and pour 2 x 25 ml water through the condenser and modified Soxhlet. Remove the reaction flask, chill under water or by surrounding with ice to solidify fats and waxes and filter off the insoluble matter on a small pledget of glass wool. Rinse the reaction flask and filter pad successively with two 10 ml portions of water. Remove the modified Soxhlet unit and wash it and the flask with hot water to remove insoluble material. Pour hot water through the condenser to remove volatile fats and oils. Discard all the washings.

Connect the flask containing the filtered sample solution to the assembled apparatus, heat and collect water and acids in the trap. Complete the digestion, using small additions of nitric acid as needed. In the final stage of digestion, adjust the heat until the digest simmers and the acid vapours do not rise above the curved half of the condenser. Continue heating for 15 minutes after the last addition of nitric acid. The digest should now be colourless or pale yellow. Let the digest cool, drain the trapped liquids carefully into the reaction flask and add 2 x 50 ml water through the condenser. Reflux solution until all NO_2 is expelled from the apparatus. Add 5 ml 40% urea solution and reflux for 15 minutes. The digest should be colourless or pale yellow.

Dried Fruit, Cereal, Seeds and Grains

Dilute the sample with 50 ml of water before adding the nitric acid, and proceed as above.

Meats, Fish and Similar Material

Conduct the initial digestion carefully to avoid foam (caused by the high fat and protein content of these materials) from reaching the condenser. Add 20 ml nitric to the sample, swirl the flask and leave to stand half an hour before heating. Add 25 ml water and heat cautiously with a small flame moved over the undersurface of the flask until the initial reaction and excessive foaming ceases. Complete the digestion as for fresh fruit.

Treatment of Digest: Titrate 1 ml of the prepared sample solution with standard alkali. Add to the whole digest the amount of concentrated ammonia solution to reduce the acidity to 1N. Swirl the flask during the addition of the ammonia to avoid local excess.

Solution must not be allowed to become alkaline as ammonia-mercury complexes would be formed. Transfer the solution to a 500 ml separator. Add 10 ml 4 mg/L dithizone solution and shake vigorously one minute. If the dithizone level remains green, less than 5 µg of mercury is present. Let the layers separate and drain the chloroform layer quickly into a second separator containing 25 ml 0.1N HCl and 5 ml of hydroxyammonium chloride solution. A small amount of oxidizing material may still be present, which could destroy the dithizone on long contact (preventing the extraction of mercury) so the chloroform layer must be run off as soon as it separates. Repeat the extraction with 2 x 5 ml of dithizone solution, transferring the chloroform layer to the second separator each time. If the first extraction indicates more than 5 micrograms of Hg use more dithizone according to the following table, until (after shaking vigorously for 1 minute) the chloroform layer remains green. Drain the chloroform layer into the second separator containing 0.1N HCl and again extract the sample with 2 x 10 ml of mg/L dithizone solution, draining each successive extract into the second separator.

Mercury Range (micrograms)	Dithizone Concentration (mg/L)	Volume of Dithizone (ml)
0 - 10	6	5
10 - 50	10	25
50 - 100	10	40

Shake the contents of the second separator vigorously 1 minute and drain the chloroform layer into a third separator containing 50 ml of 0.1N HCl. Shaking the dithizone extract with dilute acid in the second separator removes entrained organic matter. With meat and other materials of high protein content the aqueous layer is usually light yellow because of nitrated organic compounds. Small amounts are carried into the third separator where they are destroyed by chlorine. Extract the solution in the second separator with 1-2 ml chloroform and transfer the organic layer to the third separator.

To the third separator add 3 ml 30% acetic acid and the appropriate volume and concentration of dithizone solution indicated by the table. Proceed as for standards, measuring the absorbance A at 490 nm and determining the number of micrograms of mercury present from the standard curve.

To the contents of the third separator add 2 ml of 1.5% sodium thiosulphate solution, shake vigorously 1 minute and leave to separate. Drain off the chloroform as completely as possible and discard. (Copper, if present, is removed as its dithizonate). Extract again with 1-2 ml of chloroform, drain carefully and discard. Add 3.5 ml of 5% sodium hypochlorite solution (or enough of a different concentration to provide 175 mg available chlorine) to decompose the mercury thiosulphate complex and oxidise the excess thiosulphate, and shake vigorously 1 minute. Add 5 ml of hydroxylamine hydrochloride solution by pipette, taking care to wet both the stopper and the neck of the separator. Shake vigorously 1 minute. Blow any remaining chlorine gas out of the separator by a gentle jet of air. Stopper the separator and shake vigorously 1 minute. It is imperative that all hypochlorite is reduced. Trace amounts remaining would oxidize the dithizone added later, to a yellow oxidation product which would be measured as mercury. Extract the solution with 2-3 ml of chloroform, drain off the organic layer carefully and discard. The final aqueous solution so obtained should be colourless.

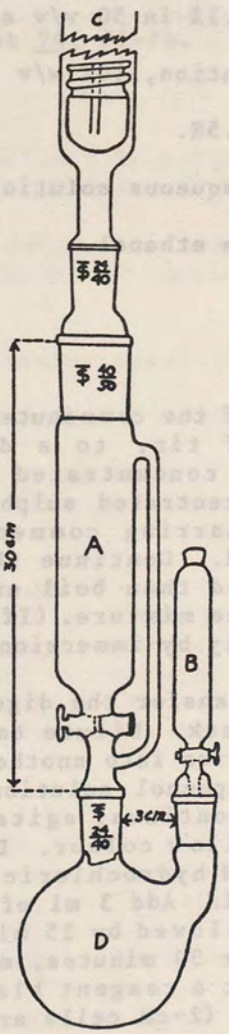
Prepare a standard curve in the required range 1-10, 1-50 or 1-100 micrograms of mercury using a blank, the standard at the top of the range and 4 intermediate standards. Add each standard and the blank to separators each containing 50 ml 0.1N HCl. Add 5 ml hydroxyammonium hydrochloride reagent and 5 ml chloroform and shake vigorously one minute. Let the layers separate, drain off the chloroform, being careful to remove all droplets of it, and discard. Add 3 ml 30% acetic acid and the appropriate volume of dithizone solution, shake vigorously one minute and let the layers separate. The acetic acid aids in stabilizing the mercuric dithizonate. Insert a cotton pledget into the stem of the separator and collect the dithizone extract (discarding the first ml) in a 1 cm cell. Read immediately at 490 nm. Both dilute dithizone and mercuric dithizonate are somewhat unstable. Plot absorbance (A) against micrograms of mercury.

CALCULATIONS

Read the micrograms of mercury for the sample absorbance, from the standard curve. Divide by the grams of sample taken to obtain ppm.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 25.138-.145.



Special Digestion Apparatus for Inorganic Mercury

TIN
(Colorimetric Method)

PRINCIPLE

The sample (containing up to 1 mg of tin) is digested in nitric and sulphuric acids and the digest diluted to a known volume. An aliquot is neutralized using 2,4-dinitrophenol as indicator, the solution acidified with a standard amount of acid and the colour with quercetin developed in 50% ethanol. Comparison of the colour is made with standard solutions developed at the same time.

APPARARUS

1. Spectrophotometer, preferably with 2 and 4-cm cells.

2. Digestion flasks of the Kjeldahl type. If silica flasks and a Meker burner are used, the digestion time can be greatly reduced (e.g. 5 g of meat can be digested in 15 minutes).

REAGENTS

1. Nitric acid (SG 1.42).

2. Sulphuric acid (SG 1.84).

3. 2,4-Dinitrophenol, 0.1% in 50 v/v aqueous alcohol.

4. Sodium carbonate solution, 10% w/v in water.

5. Hydrochloric acid, 2.5N.

6. Thiourea, saturated aqueous solution.

7. Quercetin, 0.2 w/v in ethanol.

8. Ethanol, 95%.

PROCEDURES

Transfer a known weight of the comminuted sample, containing not more than 1000 micrograms of tin, to a digestion flask (preferably silica). Add 10 ml of concentrated nitric acid and, after 10 minutes, add 5 ml of concentrated sulphuric acid. Boil the mixture vigorously and, when charring commences, add small amounts of concentrated nitric acid. Continue the oxidation in this manner until charring ceases and then boil until white fumes of sulphur trioxide appear. Cool the mixture. (If silica flasks are used this is effected instantaneously by immersion in cold water).

Add 20 ml of water and transfer the digested solution quantitatively to a 50 ml volumetric flask. Dilute to volume with water and mix. Pipette 2 ml of this solution into another 50 ml volumetric flask and add 0.2 ml of 2,4-dinitrophenol solution. Add 10% sodium carbonate solution dropwise with continual agitation of the flask until the first appearance of a yellow colour. Discharge this colour by the dropwise addition of 2.5N hydrochloric acid and immediately add a further 5.0 ml of the acid. Add 3 ml of thiourea solution and 5.0 ml of quercetin reagent, followed by 25 ml of ethanol; dilute to 50 ml with water and mix. After 30 minutes, measure the absorbance in a 4-cm cell at 437 nm against a reagent blank solution produced in the same manner as the sample (2-cm cells are preferable if the tin level is high).

Prepare a standard curve as follows. Prepare a standard tin solution by dissolving 0.0500 g of pure tin in 50 ml of boiling concentrated sulphuric acid. Cool and cautiously add this to 120 ml of water, with further cooling and transfer to a 200 ml calibrated flask. Make up to the mark with 25% v/v sulphuric acid (1 ml = 25 micrograms of tin). To a series of digestion flasks containing 5 ml of concentrated sulphuric acid and 10 ml of concentrated nitric acid, add 0, 1.0, 2.0, 3.0 and 4.0 ml of the standard tin solution. Boil until the nitric acid is expelled and white fumes apppear. Cool, add 20 ml of water and proceed with the colour development exactly as above. Construct the standard curve of absorbance against micrograms of tin.

CALCULATIONS

$$\text{Tin ppm} = \frac{A}{W}$$

where:

A = µg of tin on standard curve corresponding to sample absorbance

W = g sample

REFERENCE

Kirk and Pocklington, 1969. Analyst 94, 71-74.

COPPER
(Colourimetric Method)

PRINCIPLE

Sodium diethyldithiocarbamate is used to form a coloured complex which is then extracted with chloroform from an ammonical solution (pH 8.5) containing EDTA to prevent interference by other metals. The absorbance of the complex is measured at 440 nm.

APPARATUS

1. Spectrophotometer.

REAGENTS

1. Nitric acid, concentrated.

2. Hydrochloric acid, concentrated.

3. Citric acid, 60% w/v : Dissolve 60 g of citric acid in 70 ml of water and dilute to 100 ml.

4. Ammonium hydroxide.

5. Disodium ethylenediamine tetra-acetic acid (EDTA) 1% : Dissolve 5 g of the salt in water to dilute to 500 ml.

6. Chloroform.

7. Standard copper solution (0.5 mg/ml) : Boil 0.50 g of copper in 20 ml (1+1) nitric acid, cool and make up to 1L.

8. Dilute copper standard solution: Dilute 10 ml of the above concentrated standard solution to 500 ml to obtain 10 µg/ml. (Make fresh daily.)

9. Sodium diethylcarbodithioate solution: 0.1% aqueous solution.

10. Anhydrous sodium sulphate, granular.

11. Thymol blue indicator - 0.1% in water (add sufficient 0.1N NaOH to change the colour to blue.

PROCEDURE

Ash 20 g of sample at about 600°C in a silica dish. Extract the ash by adding 10 ml of a solution of hydrochloric-nitric-water (2+1+3) and make up to 100 ml with water. Transfer a suitable amount of aliquot into a 125 ml separating funnel and carry out the determination as in the standard curve preparation below.

Preparation of Standard Curve

Add 20 ml water to each of six 125 ml separatory funnels. Pipette 0, 1.0, 2.0, 3.0, 4.0 and 5.0 ml respectively, of the dilute standard solution into the six funnels.

Add 5 ml of citric acid and 5 ml of EDTA solutions to each funnel. Add 2 drops of thymol blue to each and then ammonium hydroxide until the solution turns green or blue-green. Next, add 10 ml of 0.1% sodium diethylcarbodithioate. Finally, add 10 ml of chloroform and

shake for about 1 min. Allow the two layers to separate and transfer the bottom layer into a 50 ml volumetric flask (filter through some cotton in a funnel, to remove water drops).

Repeat the extraction twice with 15 ml portions of chloroform (or until the chloroform is colourless). Combine the extracts and dilute to the mark with chloroform.

Measure the absorbance at 440 nm in a 1-cm cell against a reagent blank carried through the procedure. Construct the standard curve by plotting absorbance against concentration.

CALCULATION

$$\text{Copper ppm} = \frac{(A)\ (100)}{(W)\ (V)}$$

Where:

A = µg copper from standard curve, corresponding to the sample absorbance.

W = g sample

V = ml of sample extract used.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 25.066-.071.

METALS SCREENING TEST
(Lead, Copper, Zinc and Others)

PRINCIPLE

The sample is ashed or digested in acid and the solution obtained is tested for lead using dithizone and for copper using sodium diethyl carbodithioate. Another aliquot of the solution is tested with dithizone in chloroform, and a positive result is obtained in the presence of zinc and other metals such as cadmium, bismuth, copper, mercury, nickel, silver, thallium and tin. Samples giving positive results certainly require further examination by a specific and more accurate and precise method. If the positive result in the zinc test proves to be due to another element, this must be identified and quantified. This screening test does not obviate the need to test for toxic metals other than lead, copper and zinc. Although mercury would react positively in the reaction for zinc it is likely to have been largely lost during the ashing or wet digestion used.

APPARATUS

1. Muffle furnace or apparatus for digestion using Kjeldahl flasks.

REAGENTS

1. Ash aid Solution: Dissolve 50 g ammonium nitrate, and 50g potassium sulphate in 250 ml water. Check for absence of metals. Use 2 ml as an ash aid.

2. Ammonium citrate, 10%.

3. Hydroxylamine HCl, 1%.

4. Potassium cyanide, 5%.

5. Dithizone solution, 0.02% prepared from 0.2% solution in chloroform.

6. Sodium diethylcarbodithioate, 0.1%.

7. Concentrated nitric and sulphuric acids as required for digestion.

8. Standard metals solutions. Prepare aqueous solutions using pure salts in the following concentrations:

Lead	-	1 mg/ml
Copper	-	0.1 mg/ml
Zinc	-	0.1 mg/ml

9. Mixed metals standard solution: Prepare by diluting the following volume of the above solutions to a total of 500 ml in one flask: Lead - 1 ml, copper - 100 ml and zinc - 50 ml.

10. Hydrochloric acid, approximately 3N.

PROCEDURE

Prepare the sample solution by either acid digestion or dry ashing as follows:

Digestion

Weigh an appropriate amount of sample into a Kjeldahl flask and add 10 ml nitric acid. Boil over small flame until volume in the flask is reduced to 2-3 ml or until mixture becomes viscous. Do not allow charring to occur. Allow to cool, then add 10 ml concentrated sulphuric acid, mix and boil until white fumes are evolved or charring occurs, but prevent overheating. Treat blank similarly.

Place 25 ml nitric acid into a tap funnel and drop the acid into the boiling liquid in flask until the solution becomes colourless or nearly colourless. Stop heating and calculate volume of nitric added. Where more than one sample is being tested, make the volume of nitric in each flask (including the blank) up to the highest volume of nitric required. Boil down until white fumes are evolved. Allow to cool, add 10-15 ml water and again boil down until white fumes are evolved. Repeat the boiling down procedure after the addition of a second 10-15 ml water.

Cool, transfer solution using metal-free distilled water to 100 ml volumetric flask. Add 10 ml 3N HCl to Kjeldahl flask, bring to the boil, transfer to the volumetric flask, and rinse with 1 ml 3N HCl. Cool and dilute to 100 ml. Treat the blank similarly throughout. Solutions of sample and blank containing 10% sulphuric (with a little HCl) are thus obtained. Aliquots of these solutions are used for the estimations of toxic metals.

Ashing

Weigh 10 g of sample into a silica dish. Add 2 ml of ash-aid solution, thoroughly mix, dry and ash at 500°C. Dissolve in 2 ml of 3N HCl, boil and dilute to 20 ml. Traces of carbon need not be filtered.

Test for Lead

To four clean test tubes (150 x 25 mm) individually add: (1) appropriate volume of sample solution; (2) same volume of digestion or ash blank solution; (3) same volume of 10% sulphuric acid; and (4) same as (3) plus 5 ml mixed standard metal solution. In a series of samples only one of each of (2), (3) and (4) is necessary.

To each tube add 2 ml 10% w/v citric acid and mix; 0.3 ml thymol blue indicator and mix; concentrated ammonia to full blue colour of indicator and mix; 2 ml potassium cyanide and mix; 10 ml chloroform; 0.6 ml 0.02% w/v dithizone (freshly prepared). Shake each tube vigorously 10-20 seconds and allow layers to separate. The Colorimetric Blank should be green and the Standard purple-red but not full red. Record the colour of (1) and (2) as proportions of (4). Where a colour shows no difference from (3) record "less than 0.1 times Standard". If colour is more red than (4) repeat the estimation on a smaller aloquot.

(Note: Dithizone solutions may vary in strength, and the proportion of dithizone solution given above may be insufficient to give correct colour. The standard must be slightly purple indicating a slight excess of unchanged dithizone, and not a full red colour. If further dithizone is required to produce a satisfactory coloured Standard the same amount must be added to all tubes.)

(Note: For 50 ml aliquots, which are sometimes required for foods with low lead limits, the above solutions and additions and shaking should be made in a 100 ml flask. Allow the layers to separate, decant 10-15 ml of aqueous layer to waste, and pour remainder of

liquid into 150 x 25 mm tubes for comparison of colours of chloroform layer. This procedure is only necessary for 50 ml aliquots, as 25 ml aliquot and reagent can normally be accommodated in the 150 x 25 mm stoppered tubes.)

Test for Copper

To four clean test tubes (150 x 25 mm) individually add: (1) appropriate volume of sample solution; (2) same volume of digestion or ash blank solution; (3) same volume of 10% sulphuric acid; and (4) same as (3) plus 5 ml mixed standard metal solution. In a series of samples, only one each of (2), (3) and (4) is necessary.

To each tube add 2 ml 10% w/v citric acid and mix; 0.3 ml thymol blue solution and mix; ammonia to a full blue colour of indicator and mix (3N or concentrated depending upon amount of acid to be neutralized but use same reagent for all tubes); 1 ml of 0.1% sodium dithylcarbodithioate; and 10 ml chloroform.

Shake each tube vigorously for 10-20 seconds and allow layers to separate. Record the colour of (2) as proportion of (4). When the colour shows no difference from (3) record "less than 0.1 times Standard". If colour is greater than Standard, repeat on a smaller aliquot.

Test for Zinc and Other Metals

To four clean test tubes (150 x 25 mm) add: (1) appropriate volume of sample solution; (2) same volume of digestion or ash blank solution; (3) same volume of 10% sulphuric acid; and (4) same as (3) plus 1 ml mixed standard metal solution. In a series of samples only one each of (2), (3) and (4) is necessary.

To each tube add: 2 ml 10% w/v citric acid and mix; 0.3 ml thymol blue solution and mix; concentrated ammonia to full blue of indicator and mix; 15 ml chloroform; and 1.5 ml 0.2% w/v dithizone (freshly prepared). Shake each tube vigorously 10-20 seconds and allow layers to separate. The (3) tube should be green and (4) should be purple red but not full red. Record colour of (1) and (2) as proportions of (4) to 0.1. Where a colour shows no difference from (3) record as "less than 0.1 times standard". If colour is more red than (4) repeat on a smaller aliquot.

(Note: This test will include other metals; bismuth, cadmium, copper, mercury, nickel, silver, thallium and tin. If the "zinc" indicated by this estimation is less than the limit prescribed then the true zinc figure is certainly below the limit.)

7.3 Mycotoxins

Poisonous mould metabolic products are generically referred to as "mycotoxins". Several are known carcinogens or mutagens in addition to their toxicity.

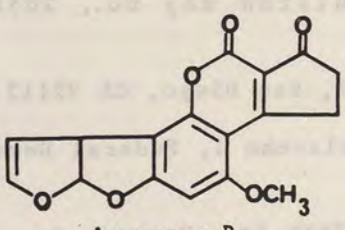
Mould growth on foods is very common, especially in warm, humid climates. It can occur in the field, or in storage after harvest. Mould infection of foods such as grains, seeds and nuts is often localized in pockets, especially in bulk storage. Frequent and adequate sampling for test, therefore becomes a necessity. The sampling problem and a solution is discussed by Whitaker et al (4), using aflatoxin in groundnuts as the example.

All food samples suspected of being contaminated with mycotoxins, must be handled with care. Use disposable gloves and protective masks if grinding the food creates dust. When handling pure mycotoxin reference material, use extreme care, preferably in a hood or microbiological glove box. Treat any spillage of toxin with a 5% solution of sodium hypochlorite and rinse all exposed glassware with a 1% solution of the bleach before washing in the usual manner. The bleach destroys the toxin by oxidation. Decontammination methods are discussed by Stoloff and Trager (5).

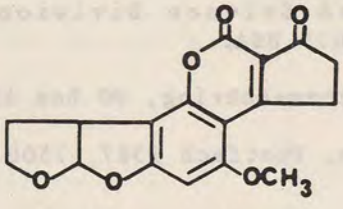
Aflatoxin

Aflatoxin is probably the most common and widely known mycotoxin contaminant. It is produced by the moulds Aspergillus flavus and Aspergillis parasiticus. In fact the name is a composite word derived from 'A. flavus toxin'. Foods which are commonly affected include all nuts (especially groundnuts and tree nuts such as pistachios and Brazils), cottonseed, copra, rice, maize, wheat grain, sorghum, pulses, figs and oilseed cakes. Unrefined vegetable oils made from contaminated seeds or nuts, usually contains aflatoxin. However, aflatoxin is destroyed in the refining process, so that refined oils are safe. It is an unfortunate fact that in many countries, unrefined vegetable oils are used in much greater quantities than refined, because of the sometimes large difference in cost. In such areas, unrefined oils should be given high priority for frequent routine testing.

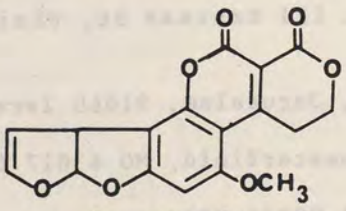
There are six aflatoxins of analytical interest. Four occur in foods and two as metabolites in the milk of animals who have been fed contaminated feed. Their chemical structures are noted below:



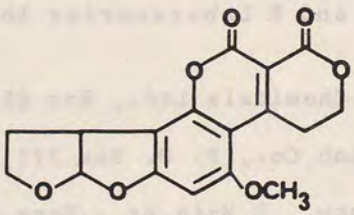
AFLATOXIN B_1



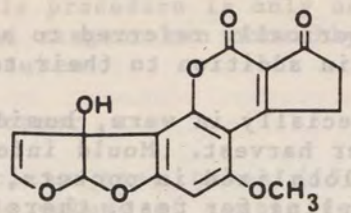
AFLATOXIN B_2



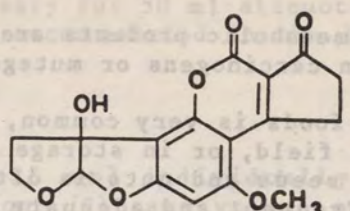
AFLATOXIN G_1



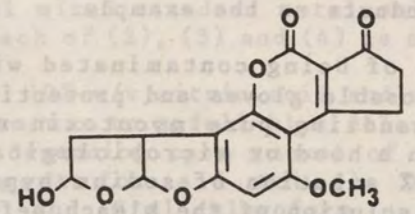
AFLATOXIN G_2



AFLATOXIN M_1



AFLATOXIN M_2



AFLATOXIN B_{2A} (WATER ADDUCT, HEMIACETAL)

Aflatoxins B_1, B_2, G_1 and G_2 refer to toxins which fluoresce blue (B) or green (G) under ultraviolet light and are separable by thin-layer chromatography. Note that the only structural difference between B and G toxins is the inclusion of an oxygen in the cyclopentanone ring. Alfatoxin B_1 is by far the most commonly found of the four toxins.

Aflatoxins M_1 and M_2 represent the toxins B_1 and B_2 which have been metabolized and converted within the body of a lactating animal. Their finding in milk led to their 'M' designation. The obvious structural difference between B and M is the addition of the hydroxyl group.

Aflatoxin B_{2A} is a hemiacetal derivative of B_1 and is commonly called the "water adduct". It does not occur naturally and is only used as a means to confirm B_1 identity.

Aflatoxins pure reference material are obtainable from the following international sources (taken from Official Methods of Analysis of the AOAC, 1984, 26.005) (list is alphabetical):

1. Aldrich Chemical Co., PO Box 355, Milwaukee, WI 53201 USA.

2. Applied Science Division, Milton Roy Co., 2051 Waukegan Rd., Deerfield, IL 60015 USA.

3. Calbiochem-Behring, PO Box 12087, San Diego, CA 92112 USA.

4. C. Roth, Postfach 1387, 7500 Karlsruhe 1, Federal Democratic Republic of Germany.

5. Eureka Laboratories, Inc., 215 26th St., Sacramento, CA 95816 USA.

6. ICN K and K Laboratories Inc., 121 Express St, Plainview, NY 11803 USA.

7. Makor Chemicals Ltd., Box 6570, Jerusalem, 91060 Israel.

8. Myco Lab Co., P. O. Box 321, Chesterfield, MO 63017 USA.

9. RFR Corp., 1 Main St., Hope, RI 02831 USA.

10. Rijksinstitut voor de Volksgezondheid, P. O. Box 1, 3720 BA Bilthoven, The Netherlands.

11. Senn Chemicals, Laboratorium Guido A. Senn, Postfach 2, CH-8157 Dielsdorf, Switzerland.

12. Sigma Chemical Co., Supelco Inc., P. O. Box 14509, St. Louis, MO 63178 USA.

13. TCI Tridom Chemical Inc., Hauppauge, NY 11787, USA.

AFLATOXINS
(Thin Layer Chromatography Method)

PRINCIPLE

This method may be used for the detection and determination of aflatoxin B_1, and aflatoxins B_2, G_1 and G_2, if needed, in a wide variety of foods, including nuts, grains, oilseeds, and mixed feeds. The limit of determination can be the very low ng/g range, dependent on whether and which optional cleanup procedures are used.

Aflatoxins are extracted from the finely ground food with aqueous methanol and partitioned to methylene chloride. For some products, such as cottonseed and mixed feeds which contain interfering pigments, an optional cleanup of the aqueous methanol extract by treatment with metals is done before the partition. If necessary, the aflatoxins are further separated from most interferences by silica gel column chromatography. Separation of the aflatoxins for detection and quantitation is by thin layer chromatography.

APPARATUS

1. Chromatographic columns, 300 mm x 10 mm ID with stopcock and either medium pore frit disc or glass wool plug.

2. Thin-layer chromatography apparatus including plates, spotting pipettes, developing tanks and a UV light viewing chamber.

3. Flask shaker apparatus.

4. Warm air blower (such as hair dryer).

REAGENTS

(Note: All solvents must be procured and stored in glass containers; plastic liners or coating used in metal containers contain extractives that interfere with analysis.)

1. Acetic acid

2. Acetone

3. Acetonitrile

4. Benzene

5. Chloroform

6. Ethyl ether

7. Formic acid

8. Isopropanol

9. Methanol

10. Methylene chloride (dichloromethane)

11. Petroleum ether

12. Toluene

13. Diatomaceous earth filter aid - Acid washed Hyflo SuperCel (Johns Manville Corp., Greenwood Plaza, Denver, CO 80217, USA)

14. Silica gel - Particle size 80-230 mesh

15. Sodium chloride solution, 100 g/L

16. Sodium sulphate, anhydrous, granular

17. Zinc acetate-aluminium chloride solution - 200 g $Zn(OAc)_2$ and 5 g $AlCl_3$ to make 1 L with water

18. Aflatoxin standards - prepare a mixed working standard to contain 0.5 µg/ml each of aflatoxins B_1 and G_1 and 0.1 µg/ml each B_2 and G_2.

PROCEDURE

Prepare the food by grinding or comminuting to pass a 20 mesh screen. Use as large a sample as possible depending on the available preparation equipment. (This should be as much as 10 kilos if possible. The larger the sample size, the greater the potential to detect pockets of contamination).

Weigh 50 g sample in glass-stoppered erlenmeyer flask, and add 200 ml methanol:water (85:15). Shake vigorously no less than 30 min (use mechanical shaker). Filter through medium porosity paper. Collect 40 ml filtrate from midflow, or, if optional cleanup is used, collect 1 ml filtrate from start of flow.

Optional clean-up to remove interfering plant pigments from cottonseed or mixed animal feeds: transfer 100 ml above filtrate to 250 ml beaker. Add 20 ml $Zn(OAc)_2$-$AlCl_3$ solution and 80 ml water. Stir and let stand at least 5 min to flocculate. Mix in 5 g filter aid and filter through medium porosity paper. Collect 80 ml filtrate from midflow.

Transfer either the 40 ml or the 80 ml filtrates from above to a separatory funnel. Add 40 ml sodium chloride solution and 25 ml petroleum ether. Shake 1 min, let layers separate and transfer bottom layer to second separatory funnel. Discard top layer. (Repeat this partition for high fat content foods). Add 25 ml methylene chloride to the second funnel. Shake 1 min, let layers separate and drain bottom layer into 125 ml erlenmeyer flask. Repeat the extraction with a second portion methylene chloride and combine with the first. Add several boiling chips and evaporate the combined methylene chloride extracts almost to dryness on a steam bath. Cool, and cover with aluminium foil, and hold for either thin layer chromatography or column chromatography (if needed for further cleanup of extract).

If further column chromatography clean-up is needed to remove interferences, proceed as follows: Prepare a column by slurrying 2 g (about 5 ml volume) of 80-230 mesh silica gel with 10 ml ether-petroleum ether (3+1) in a small beaker and pouring into the column. Wash any residual silica gel into the column using 5 ml of the (3+1) mixed ethers. After the gel settles, top the gel packing with 1.5 g sodium sulphate. Drain the solvent to the top of the sodium sulphate. Dissolve residue in 3 ml methylene chloride, and transfer solution to column. Rinse flask twice with 1 ml portions methylene chloride and add rinsings to column. Elute extract through column no faster than 1 ml/min. Add successively to column 3 ml petroleum ether, 3 ml ethyl ether and 3 ml methylene chloride. Elute through columns no faster than 3 ml/min. Discard eluates. Add 6 ml methylene chloride:acetone (9+1) to column. Elute solvent through column (preferably with no vacuum) no faster than 1 ml/min. Collect eluate in a small vial from time of solvent addition to cessation of

flow. Add a few boiling chips and evaporate eluate just to dryness on steam bath under a stream of nitrogen. Cap vial, wrap in foil, and hold for thin layer chromatography.

Uncap vial containing extract residue, add 0.2 ml benzene-acetonitrile (98+2), reseal vial, and shake vigorously, preferably with Vortex type mixer to dissolve residue. On a thin layer plate score a line in silica gel layer to divide plate into 2 equal 10x20 cm sections. Also score lines perpendicular to the first score line and at 1 cm intervals across the plate to produce 1 cm wide channels for spotting and development. Along one 20 cm dimension, on an imaginary line 3 cm from bottom edge of a scored plate, spot 4 centre channels with 2.5, 5.0, 7.5 and 10 µl of the mixed aflatoxin reference standard. In the remaining channels spot 2-10 µl of each sample extract and superimpose 5 µl of aflatoxin standard on 1 spot of each of the sample extracts.

Develop spotted plate until solvent reaches score line that divides plate into 2 parts (ca 20 min). Use an unequilibrated tank with solvent combination previously selected from the following for optimum resolution of aflatoxins from each other and substrate interferences: (order of aflatoxin R_f from top is B_1, B_2, G_1, G_2)

a. Chloroform:acetone (9+1)
b. Chloroform:acetone:water (88+12+1.5)
c. Chloroform:acetone:isopropanol:water (88+12+1.5+1)
d. Chloroform:isopropanol (99+1)
e. Benzene:methanol:acetic acid (90+5+5)
f. Ether:methanol:water (96+3+1)

(Note: Polar component of each combination may be adjusted to change absolute R_f. Increase the proportion for higher R_f, decrease to lower R_f).

Remove the plate from tank and evaporate solvent from the plate in a hood at room temperature. Examine plate under long-wave UV light in darkened room or a cabinet. Look in channels with reference standards for clean separation of 4 blue fluorescent spots. Look in sample channels without superimposed standard for fluorescent spots that coincide in hue and development position with reference standards in adjacent channels. Each sample-plus-standard fluorescent spot should produce a more intense fluorescence than corresponding spot from the sample alone, and should show no sign of separating spots (evidence of dissimilar compounds).

Compare the intensity of each fluorescent spot presumed to be an aflatoxin, with corresponding spots of reference standards alone in four centre channels. Look for an intensity match. Interpolate, if sample intensity lies between intensities of two standards. If spot from sample is too intense to match highest standard, estimate relative intensities, dilute sample by this factor, and rechromatograph.

CALCULATION

The mixed standard spots of 2.5, 5.0, 7.5 and 10 µl are equivalent to the following ng aflatoxins respectively:

B_1 and G_1 - 1.25, 2.5, 3.75, 5.0

B_2 and G_2 - .25, .5, .75, 1.0

The sample spots are equivalent to 0.05 g sample/µl of extract spotted. Therefore, if sample spots of 2 to 10 µl are made, these would be equivalent to 0.1 to 0.5 g.

Therefore:

$$\text{Aflatoxin ppb (ng/g)} = \frac{W}{0.05\ V}$$

where:

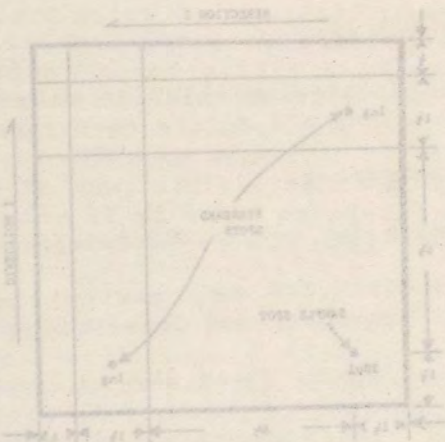
W = ng aflatoxin with closest intensity match to sample spot V.

V = µl of sample spot closest to W.

Calculate for each different aflatoxin in a given sample spot and add together so that total aflatoxins are reported. (For example: found 10 ppb B_1, 3 ppb B_2 and no G_1 or G_2 - Report as 13 ppb total aflatoxins).

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 26.001-.013.



AFLATOXIN B_1

CONFIRMATION OF IDENTITY

PRINCIPLE

Some food products may give a blue fluorescent spot similar in R_F to aflatoxin B_1 (the most commonly found aflatoxin). This method provides a means to confirm the B_1 identity by preparing the hemiacetal B_{2A} and identifying it using two-dimensional thin-layer chromatography.

APPARATUS

1. Thin-layer chromatography apparatus including plates, spotting pipettes, developing tanks and a UV light viewing chamber.

2. Warm air blower (such as a hair dryer).

REAGENTS

1. Silica gel for TLC plates.

2. Trifluoroacetic acid (TFA).

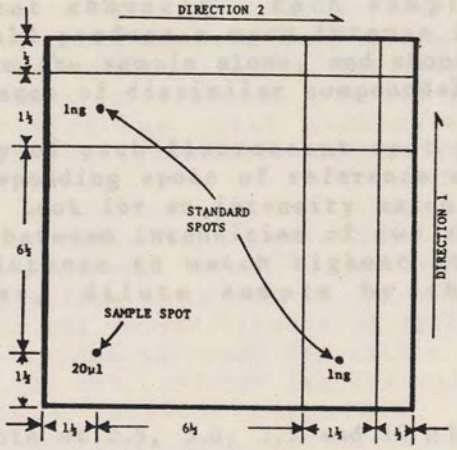
3. Developing solvent A: ethyl ether-methanol-water (96+3+1).

4. Developing solvent B: chloroform-acetone-isopropanol (85+12.5 +2.5).

5. Aflatoxin B_1 working standard of 0.5 µg B_1/ml.

PROCEDURE

Prepare silica gel TLC plates as in usual aflatoxin analysis. Score a plate for two-dimensional chromatography, as shown in the figure below. (The distances given on the figure are for a 10 cm x 10 cm plate. For a 20 cm x 20 cm, they would double to 3 cm, 13 cm, 3 cm and 1 cm respectively).



On the sample spot, apply sufficient sample extract to give an estimated 5 ng aflatoxin B_1. On both standard spots, apply 10 µl of the B_1 working standard. Overspot all three spots with 2 µl each of trifluoroacetic acid. Place plate in a dark area for 5 min to complete the reaction. Evaporate residual TFA using warm air (TFA must be completely dry before continuing).

Develop plate in direction 1 using Solvent A until the solvent front reaches the lower scored line. Remove the plate from the tank and dry with warm air or in a oven for a few minutes.

Turn plate 90° and develop in direction 2 using Solvent B, again until the solvent front reaches the lower scored line. Remove from the tank and dry.

Examine the dry plate using long wave UV light in a cabinet. The sample and standards should have a new blue fluorescent spot of B_{2A} at an R_F of about 0.3. The sample B_{2A} spot should lie at an intersection of imaginary lines projected from the two standard B_{2A} spots, perpendicular to the direction of each development.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 26.083.

AFLATOXINS
(Mini-column Method)

PRINCIPLE

The sample is extracted with acetone-water, impurities are removed, and the aflatoxins extracted into chloroform. The chloroform extract is passed through a column containing calcium sulphate, Florisil, alumina and silica gel. The aflatoxins remaining at the top of the Florisil layer are compared with standards under longwave UV.

APPARATUS

1. Food blender, or shaker.

2. Ultra-violet lamp, long-wave (365 nm). Intensity at least 430 micro-watts/cm^2 at 15 cm.

3. Minicolumn. Borosilicate tubing 6 mm ID x 190 mm long, tapered at one end to about 2 mm.

4. 5 ml syringe with 5" needle.

5. Filter paper, 24 cm, Whatman No. 4 or equivalent.

REAGENTS

1. Chloroform (technical grade is adequate).

2. Acetone (technical grade is adequate).

3. 0.2 N sodium hydroxide (8 g/L).

4. Ferric chloride solution. Mix 20 g anhydrous ferric chloride with 300 ml of water. Alternatively, dilute 33 ml of a 60% solution to 300 ml.

5. Copper carbonate, basic (cupric hydroxide carbonate) solid.

6. Diatomaceous earth.

7. Sulphuric acid, 0.03% v/v.

8. Potassium hydroxide/potassium chloride wash solution. Dissolve 1.12g KOH + 10 g KCl in water and dilute to 1 litre.

9. Sodium sulphate, anhydrous.

10. Florisil 100-200 mesh for column chromatography.

11. Silica gel 60, 0.063-0.200 mm, neutral for column chromatography, Activity 2-3 (Brockmann and Schodder).

12. Calcium sulphate anhydrous, 20-40 mesh.

13. Alumina, activity 1 (Brockmann and Schodder), 80-200 mesh, neutral.

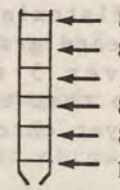
14. Aflatoxin B_1 or mixed aflatoxins standard with a final concentration of 2 µg/ml of each aflatoxin.

15. Glass wool.

16. Elution solvent. Chloroform-acetone (9+1).

PROCEDURE

Prepare one minicolumn per sample as follows: Block the tapered end of a minicolumn with a small pledget of glass wool, and fill the column as given in the diagram, tapping the column after each addition. Place a small pledget of glass wool on top and press lightly into place with a glass rod. (Note: dry each of the adsorbents for 2 hours at 110°C before use.)



Also prepare a reference minicolumn as above. To this add 5 µl of the standard solution which has been dissolved in 1 ml of chloroform. Use a syringe for the addition. As the solvent sinks to the surface of the column, add 3 ml of the chloroform-acetone (9+1) and allow to drain by gravity. If dried and sealed and kept in the dark in a freezer, the column will keep 2-3 months, but repeated exposure to ultraviolet light diminishes the intensity of the fluorescence, which is apparent at the top of the Florisil layer. This fluorescence is due to the aflatoxins.

Next, mix 50 g sample and 250 ml acetone+water (85+15) in a blender at high speed for 3 minutes or agitate on a mechanical shaker for 45 minutes. Filter through a 24 cm Whatman No. 4 filter paper into a graduated cylinder. Transfer 150 ml of the filtrate to a 400 ml beaker, add about 3 g of basic copper carbonate and mix well. To a 500 ml beaker add exactly 170 ml of 0.2N NaOH and 30 ml ferric chloride solution and mix well. Pour the mixture of filtrate and basic copper carbonate into this, add 150 ml (use a 150 ml beaker as a scoop) of diatomaceous earth and mix well. Filter through a Whatman No. 4 or equivalent filter paper.

Transfer exactly 150 ml of filtrate to 500 ml separator, add 150 ml of 0.03% sulphuric acid solution and 10 ml chloroform. Shake vigorously 2 minutes and allow to separate. Transfer the lower layer to a 125 ml separator, add 100 ml potassium hydroxide/potassium chloride wash solution, swirl gently half a minute and allow to separate. If an emulsion is formed, drain it into a lipless graduated cylinder, add about 1 g anhydrous sodium sulphate, stopper, shake half a minute and allow to separate. It is not important that the chloroform layer be completely clear. If the emulsion is still not broken, wash it with 50 ml 0.03% sulphuric acid in a separator. Finally, collect at least 3 ml of the chloroform layer in a 10 ml graduated cylinder.

By means of a 5 ml syringe with a 5 in needle, transfer 2 ml of the chloroform extract to a minicolumn. Allow to drain by gravity. The solvent may be forced through the dry column at a rate not exceeding 10 cm/min until it reaches the tip, by application of gentle air or inert gas pressure to the top of the column. Then allow to drain by gravity. When chloroform reaches the column surface, add 3 ml of elution solvent chloroform+acetone (9+1) and allow to drain by gravity.

Examine the sample and reference columns side by side in a UV cabinet. The latter has a blue fluorescent band about 2.5 cm from the bottom, at the top of the Florisil layer. A similar blue fluorescent band in the sample column is presumptive evidence of the

presence of aflatoxins. Some samples show a faint white yellow or brown fluorescence, but if there is no definite bluish tint there are no aflatoxins.

CALCULATIONS

The reference minicolumn contains 10 ng of each aflatoxin. The extract from 50 x 150/250 x 150/350 = 13 g is shaken with chloroform. The extract contains acetone, so although only 10 ml of chloroform is added, the non-aqueous layer is about 13 ml. The 2 ml of chloroform applied to the minicolumn therefore contains aflatoxins from about 2 g of sample and sample columns showing fluorescence greater than that of the reference are likely to contain over 5 ng/g (ppb) of aflatoxin. The sample column may be retained so that the presumptive aflatoxin can be eluted and examined by thin layer chromatography.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 26.014-.019.

AFLATOXIN M
(Thin-layer Chromatography Method)

PRINCIPLE

Aflatoxin M is extracted from milk or milk products and isolated by partitioning and later column chromatography. Final determination is by thin-layer chromatography.

APPARATUS

1. Explosion-proof blender with 1 L jar and cover.

2. Chromatographic tubes, 22 mm (ID) x 500 mm with stopcock.

3. Thin-layer chromatography apparatus, complete.

REAGENTS

1. Acetone

2. Benzene

3. Chloroform

4. Ethyl ether

5. Hexane

6. Methanol

7. Isopropanol

8. Lead acetate solution - dissolve 200 g lead acetate trihydrate in water, add 3 ml acetic acid and dilute to 1 L.

9. Sodium sulphate solution, saturated - dissolve 150 g anhydrous sodium sulphate in 500 ml water. (Prepare 2 or 3 days in advance of analysis).

10. Diatomaceous earth.

11. Cellulose - Whatman CF-11 or CF-1 powder - soak 4 hr in hot chloroform, filter and wash with chloroform, and air dry. Check each prepared lot for M recovery using a standard M solution through the method.

12. Aflatoxin M standard solution, 0.5 µg/ml.

PROCEDURE

Weigh fluid or powdered milk or butter into blender jar and add the amount of water indicated in the table below. Also add 10 g diatomaceous earth and 300 ml acetone. For cheese, weigh then cut into small cubes and add to the water, acetone and diatomaceous earth already in the blender jar.

Table of water addition:

Milk product	Sample volume or weight	ml water to be added
Fluid milk	100 ml	10
Powdered milk	10 g	100
Blue cheese	50 g	80
Ricotta cheese	50 g	65
Cheddar cheese	50 g	80
Butter	50 g	90

Blend 3 min then filter through folded Whatman 2V paper (or equivalent) into a 500 ml graduated cylinder. Transfer 275 ml into a 600 ml beaker containing 20 ml lead acetate solution. Rinse the transfer cylinder with 200 ml water and add to the beaker. Stir and let stand 5 min for precipitation to take place. Add 10 ml saturated sodium sulphate and 10 g diatomaceous earth. Stir and filter into a 500 ml cylinder.

Transfer 350 ml filtrate to a 500 ml separator and add 100 ml hexane (for butter or annatto coloured cheese, use ether). Shake vigorously 1 min and let stand. Drain lower aqueous layer into 600 ml beaker. Discard upper hexane or ether layer. Pour aqueous layer back into the same separator. Rinse the beaker with 50 ml 5% sodium chloride solution and add to the separator. Add 100 ml chloroform and shake 1 min. Let stand, then drain lower chloroform layer into a 600 ml beaker and re-extract the aqueous layer with an additional 50 ml chloroform. Combine the chloroform extracts. Discard the aqueous layer.

Transfer the chloroform extracts back to the separator. Rinse the beaker with 100 ml 5% salt solution and add to the separator. Shake 1 min and drain lower chloroform layer through 5 cm of anhydrous sodium sulphate into a 400 ml beaker. Discard the aqueous phase. Evaporate chloroform on a steam bath under a gentle stream of nitrogen (vacuum evaporation may be used - do not overheat the dry extract).

Prepare a chromatographic column by first inserting a glass wool plug in the bottom, then adding a slurry of 10 g cellulose powder in 70 ml methanol-water (7+3). Wash the walls of the column with more methanol-water and drain the solvent until it reaches the top of the cellulose. Next add 100 ml hexane and drain off 50 ml. Place a plug of glass wool on top of cellulose, tamp firmly to pack (prevents channeling). Drain remaining hexane to top of cellulose.

Dissolve the dry extract with 1 ml chloroform and add 2 ml benzene and 10 ml hexane. Transfer to the prepared column. Wash the empty sample beaker with 5 ml hexane-benzene (3+1) and add to column. Drain column until sample solution reaches top of glass wool. Add a wash of 150 ml hexane-benzene (3+1) and then 150 ml hexane-ether (2+1) (let the first drain to the top of the glass wool before adding the second). Discard all column washes.

Elute aflatoxin M with 200 ml hexane-chloroform (1+1) and collect eluate in a 150 ml beaker. Evaporate on a steam bath as before. Dissolve residue in a small amount of chloroform and transfer quantitatively to a small glass vial. Evaporate to dryness on a steam bath using nitrogen.

Add 100 µl chloroform (microlitre syringe is convenient) to the vial. Cap and shake 1 min to dissolve residue. Prepare TLC plates as for regular aflatoxin analysis. Spot two 20 µl sample spots plus 2, 4, 6, 8 and 10 µl M standard. Spot a 4 µl M standard over one sample spot to serve as a control. Develop the plate using isopropanol-acetone-chloroform (5+10+85). Visualize the spots using longwave UV light in a cabinet.

CALCULATION

By following the above method exactly, the following sample equivalents will be in the final extract:

Fluid milk	47.6 ml
Powdered milk	4.8 g
Cheeses and butter	23.8 g

$$\text{Aflatoxin M ppb} = \frac{S \times 0.5 \times 100}{W \times 20}$$

where:

S = µl of standard spot equivalent to sample

W = sample equivalent from above.

REFERENCE

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8. COMPOSITIONAL ANALYSIS METHODS

Compositional analysis is more commonly referred to as "proximate" analysis. It refers to the determination of the major constituents of a food, and is used to assess if the food is within normal compositional parameters, or has somehow been adulterated.

The four usual constituents measured for compositional analysis are:

Moisture
Fat
Protein
Ash

Other constituents such as fibre and added substances such as salt and starch also may be determined to give further information on food composition, especially for processed foods.

8.1 Moisture

Water is often one of the major constituents in a food. It is held to other constituents by physical and chemical forces of diverse nature and strength and its determination is often subject to a number of inaccuracies. The variety of ways in which water is present in food include: as a solvent or dispersion medium, as mono- or polymolecular layers or in capillaries, held by molecular forces, or combined as water of hydration, either as true hydrates or held by hydrogen bonding within protein and polysaccharide molecules. Water is not released from these different states with equal ease and therefore different and to some extent arbitrary methods are used with different foods.

The two most common moisture determinations are direct drying and co-distillation. Chemical methods such as Karl-Fischer titration will not be discussed here.

In direct drying, the presence of other volatile substances and chemical reactions leading to the creation of water and other volatile substances will cause false high results. False low results may occur due to incomplete removal of water under the drying conditions chosen. If drying is continued for too long a period there may be an actual increase in weight due to oxidation. The result will also vary with the extent to which the cell structure is broken down as well as the particle size. There are many possible sources of error. For routine moisture determinations at ambient pressure, use an oven with air circulation. Dishes of aluminium, nickel, stainless steel or platinum are suitable. Moisture determinations carried out in silica tend to be less accurate as moisture may be picked up during cooling prior to weighing. On the other hand, it is sometimes convenient to determine the ash subsequently in which case silica, porcelain or platinum must be used. A well-fitting lid is essential for samples that are hygroscopic once dried (such as milk and cereals). The lid is placed on the dish just prior to the removal of the latter from the oven. The cooling desiccator must contain effective desiccant, self-indicating silica gel being convenient. An hour or two in the oven is sufficient to regenerate this. Dishes must be left in the desiccator long enough for the moisture film on the outside of the dish to reach equilibrium with the surroundings. This may take 30-40 minutes. Samples that contain fat or can form an impervious skin are dried on sand. The sand is prepared by boiling with 50% hydrochloric acid until there is no further extract and washing free of chloride with water.

Standard methods for moisture determination by oven drying stipulate drying at a certain temperature either for a specified time or until constant weight is achieved. In the latter case, the sample is removed after a few hours, weighed, and returned to the oven for approximately one hour periods until successive weighings do not differ by more than a small amount (0.5 mg, 1 mg, 2

mg or 5 mg varying with the method and the material). Even if the differences exceed the specified value it is probably not worthwhile to continue drying once the weights in each successive heating are not decreasing significantly.

Drying under vacuum at reduced pressure is used for products such as fruit and vegetables where chemical reactions and the presence of volatile substances would give rise to serious errors at a higher temperature. Vacuum must be obtained and then water released slowly enough so that loss from the dish does not occur. There must be a dry air-bleed passing through the oven. The air is taken from a cylinder or may be conveniently dried by bubbling through concentrated sulphuric acid. Two pressures are commonly used, around 100 mm Hg, or around 25 mm Hg. Under the S.I. system of units pressure is expressed as pascals (Pa). 25 mm Hg is equivalent to 3.3 kPa. In some methods, the pressure is expressed as millibars (1000 millibars equal 760 mm Hg).

There are a number of rapid thermogravimetric methods for determining moisture. These have often been developed in response to the needs of industry for rapid quality control procedures. Provided results are reproducible, corrections can be made for known inaccuracies. Infra-red moisture testers and the Brabender moisture oven for flour samples are examples of equipment used for this type of method.

Azeotropic co-distillation is often used for foods containing volatile oils. A water-immiscible organic solvent (often toluene) is boiled with the food and water vapour distils over, being collected in a graduated trap. The process tends to be incomplete so that results may be low. The organic solvent should be shaken with a small quantity of water and distilled prior to use. The upper parts of apparatus may be wrapped in an insulating material in order to obtain a more even heating. A spiral copper wire passed down the condenser will dislodge any water adhering to the walls. Occasional addition of 5 ml portions of toluene down the condenser may also assist in this. Refluxing is continued until the water level in the receiver remains unchanged for 30 minutes and the reading taken 20 minutes or so after removing the source of heat. The determination should be carried out in a fume hood due to the toxic nature of the solvents and the form of heating must not be a naked flame, due to the fire risk. If difficulty is experienced with droplets of water adhering to the walls of the condenser, the cooling water may be turned off for a few minutes before the end of the distillation. Cleaning the apparatus, including the condenser, in chromic acid prior to use is also helpful. The rate of distillation should be about 100 drops per minute, increasing to about 200 drops per minute near the end of the heating period. Heating for an hour or longer may be necessary to distil all the water.

The resistance of foods to the passage of an electric current varies with the amount of water present. The effect has been used as the basis for the design of several moisture meters. Moisture meters depending on the conductivity principle have means for compressing the sample in a chamber of definite size to achieve adequately consistent results. The area of contact between the conducting particles and hence the particle size have an important effect in the conductivity of the mass. The moisture also has to be evenly distributed throughout the food in order to obtain reliable results.

MOISTURE
(Air Oven Method)

PRINCIPLE

The sample is dried at 100-102°C for 16-18 hours or at 125°C for 2-4 hours in a forced draft air oven. The loss in weight is reported as moisture. This method is applicable to meat and fish, and other products. If the product is a cereal, heat for one hour at 130°C. If it is a dried vegetable or tea, heat in a vacuum oven for five hours at 100°C using 100 mm of mercury. If it is a spice or is expected to contain volatile oils, use the toluene distillation method.

APPARATUS

1. Forced draft air oven maintained at appropriate temperature.
2. Moisture dishes.
3. Desiccator containing absorbing material (e.g. Silica gel).
4. Analytical balance.

PROCEDURE

Reduce sample to fine form and mix well. Weigh accurately 3-4 g of the sample (in duplicate) into moisture dishes. (Sample should be spread evenly across the dish and weighed as rapidly as possible to minimize loss of moisture). Dry the sample for 16-18 hours at 100-102°C, or for 4 hours at 125°C. (Care must be exercised that drying oven is not overloaded or samples will be insufficiently dried and lower results will be obtained.) After the drying is complete, remove samples from the oven and place in desiccator. Cool to room temperature (for about 30 minutes) and weigh accurately.

CALCULATION

$$\text{Moisture (\%)} = \frac{(B - C) \times 100}{A}$$

Where A = sample weight in g.

B = weight of dish + sample prior to drying

C = weight of dish + sample after drying

(B-C) = loss in weight of sample after drying

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 24.003.

MOISTURE
(Toluene Distillation Method)

PRINCIPLE

This method is applicable for most dried products with a moisture content greater than 1%. It has been found very useful for the determination of moisture in milk powders and in grain products. The method is of special importance when it is desired to determine water in spices or other foods which contain volatile oils. Toluene is added to a sample in a flask and heated to boiling. The water and toluene boil off as an azeotropic mixture. Upon cooling the water and toluene separate and the water is collected in a special trap and measured. Benzene is used instead of toluene on red peppers, chili powder and paprika which tend to decompose at 100°C to release water.

APPARATUS

1. Balance - approximately 500 g capacity and 0.1 g or better sensitivity.

2. Condenser - Liebig, 500 mm in length.

3. Condenser brush - overall length 24", length of bristle tuft 3 diameter of tuft.

4. Distillation traps - Dean and Stark or Bidwell-Stelling type (5 ml graduated in 0.1 divisions).

5. Electric heater - with rheostat control to maintain a distillation rate of approximately 4 drops/sec under conditions of the test.

6. Flask - Erlenmeyer or round bottom, 300 ml narrow-mouth.

REAGENTS

1. Toluene (technical grade) (Blank determinations should be made.)

PROCEDURE

Accurately weigh an appropriate amount of sample (see below) and as quickly as possible transfer to a clean, dry 300 ml Erlenmeyer or round bottom flask.

Moisture Estimate	Sample Size
5%	50 g
5 - 10%	30 g
10 - 25%	20 g

Immediately pour enough toluene to cover sample completely (about 100-150 ml). Rinse down any particles on the side of the flask when introducing the toluene. Swirl the flask with sample thoroughly. Connect the flask to the distillation trap and the condenser. Make sure that all joints are tight. Fill receiving tube with toluene, pouring it through the top of the condenser. Heat the contents to boiling but make sure that the sample does not scorch on the bottom of the flask (see that cold water is circulating through the condenser). The amount of heat should be so regulated that the toluene will condense into the trap at a rate of 4 drops/sec. This

rate is maintained throughout the remainder of the distillation. (Most of the water in the sample will pass over to the trap within the first 15 min).

When all the water is apparently in the trap (usually about 45 min after distillation has begun) and without interrupting distillation, dislodge water droplets in the condenser tube by means of a condenser brush. While the brush is in the upper port of the condenser, flush the tube with about ten ml of toluene. If any water adheres to the side of the tube (trap), rub it down by means of a long, stiff wire. Read the moisture level in the trap to the nearest half a scale division (0.05 ml). Continue the distillation for an additional 30 min to see if more water distils over. If so, repeat washing-down process and note the water level in the trap. If this reading agrees with the previous reading within 0.05 ml water in the trap, discontinue the distillation. (For some products, a 75 min distillation period is not sufficient. If readings fail to agree as required, continue the distillation for additional 15 min periods until successive results agree within a 1/2 scale division). Record the amount of water collected in the trap.

CALCULATION

$$\% \text{ moisture in sample} = \frac{V \times 100}{S}$$

Where: V = volume of water in trap (in ml)

S = sample weight (g)

INTERPRETATION

It is extremely important to clean the condenser and distillation trap thoroughly in order to minimize the adherence of water to the glass surface during distillation. If equipment is dirty or greasy, difficulty will be experienced in dislodging water droplets and results may be unreliable. Wash glass equipment by soaking in and scrubbing with a solution of suitable detergent; then rinse with clean water. If this fails to remove dirt and grease, soak glassware overnight in chromic acid cleaning solution and rinse equipment well with clean water and dry thoroughly. Distillation traps require thorough cleaning after each determination and condensers usually once a week.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 7.004-.005.

MOISTURE
(Vacuum Oven Method)

PRINCIPLE

This method is applicable especially to processed vegetables. The ground or comminuted sample is first partially dried, by one of three techniques, then is vacuum dried.

APPARATUS

1. Sieve with square opening 2.8 x 2.8 cm (11.2 x 11.2 for tomato), 20 cm diameter if total contents of sample less than 1.5 kilos, 30 cm if more.

2. Food chopper or blender.

3. Water-bath or forced draft oven.

4. Vacuum oven at 70°C with temperature variation on different parts of the shelf not exceeding about 2°C.

PROCEDURE

For foods composed of solid and liquid portions (e.g., canned vegetables) if only the solid portion is required for analysis, thoroughly grind the drained food in a mortar or food chopper. However, if a composite of solid and liquid portion is required, grind the entire contents of the can in a mortar or food chopper. In all cases, thoroughly mix the portion used and store the remainder in a glass-stoppered container.

For comminuted products (tomato juice, tomato catsup, strained vegetables) thoroughly shake the unopened container to incorporate any sediment. Transfer entire contents to large glass or porcelain dish, and mix thoroughly, continuing stirring 1 minute. Transfer the well-mixed sample to a glass-stoppered container and shake or stir thoroughly each time before removing portions for analysis.

To a flat-bottomed metal dish with a tight-fitting cover, add about 15 mg of diatomaceous earth filter-aid/square cm, dry about 30 minutes at 110°C, cool in desiccator, weigh, and to each dish add a sample of such size that the dry residue will be over 9 but less than 30 mg/sq cm. Weigh as rapidly as possible to avoid moisture loss. Mix with filter-aid and distribute uniformly over the bottom of the dish, diluting with water if necessary to facilitate distribution. Bring the sample to apparent dryness (remaining moisture not more than about 50% dry solids) by one of the following methods:

(a) Place samples on a boiling water-bath and remove when samples reach apparent dryness.

(b) Place samples in a forced-draft oven at 70°C. The oven must have rapid air circulation and enough exchange with outside air to remove the moisture rapidly. Examine the dishes at intervals of 30 minutes and remove as soon as they reach apparent dryness.

(c) Place samples in a vacuum oven at 70°C with the vacuum leak-valve left partly open to allow a rapid flow of air through the oven at 310 mm Hg. Examine the dishes at 30 minute intervals and remove when they reach apparent dryness.

Place the partially dried samples in a vacuum oven with the bottoms of the dishes in direct contact with the shelf. Measure the temperature of the oven by a thermometer in direct contact with the shelf. Admit dry air to the oven at a rate of 2 - 4 bubbles/second by bubbling through sulphuric acid. Dry the samples for 2 hours at 69 - 71°C (oven may be as low as 65°C at the start of the drying, but must reach 69 - 71°C before the end of the first hour) at a pressure of 50 mm Hg. (As the dried sample will absorb an appreciable amount of moisture on standing over most desiccating agents, cover quickly and weigh as soon as possible after the sample reaches room temperature.)

CALCULATION

$$\% \text{ total solids} = \frac{\text{weight of dry residue}}{\text{weight of sample taken}} \times 100$$

The "water capacity" of a rigid container such as a can or glass jar is the volume of distilled water at 20°C which the container will hold when completely filled. In the case of cans, the lid is cut off without removing or altering the height of the double seam. The can is weighed when clean and dry, filled with distilled water at 20°C to within 4.8 mm vertical distance below the top and weighed again. The weight in grams is taken to be equal to the volume in ml (the error being less than 0.2%). The total solids may be expressed as a percentage of the water capacity by use of the following formula:

$$\text{Total solids as \% of water capacity} = \frac{\text{\% total solids in product} \times \text{net contents}}{\text{water capacity}}$$

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 14.002-.003.

8.2 Fat

The fatty material or "crude fat" in foods is frequently determined by extraction of the dried food with diethyl ether or petroleum ether or of an aqueous mixture of the food by a mixture of those two solvents, from alkali in the Rose-Gottlieb method and from acid in the Weibull-Stoldt and Schmid-Bondzynski-Ratzlaff methods. One worker recommended use of chloroform/methanol mixtures in the presence of water. This method gives significantly higher results in some cases, for example in raw lean beef. This is partly because chloroform/methanol is a more effective solvent for phospholipids and some of the other fatty substances commonly found in small amounts. In the use of this method the presence of a high polarity solvent (water, methanol) is important to disrupt hydrogen bonds and release fat bound to protein or carbohydrate. The procedure was originally intended for the extraction of total lipids from animal tissues. Although triglycerides and phospholipids are extracted from processed food, chlorine and similar compounds will not be extracted without prior splitting with hydrochloric acid as they remain incorporated in the protein matrix.

Drying of the sample prior to extraction with diethyl ether or petroleum ether may cause formation of a "skin" that retards extraction. This can sometimes be avoided by drying the sample over sand and transferring the dry material and the sand to the extraction apparatus. Plant cells containing fat will retain this fat, so that it is necessary to disrupt the cells by abrasion of the cell walls with sand after the removal of free fat and continue the extraction for a further period of time. This procedure may also augment the amount of fat obtained from samples in which the fat is bound to other constituents such as protein. Damp diethyl ether dissolves sugars and other materials to some extent and for this reason, methods consist of either extraction of the dried sample by petroleum ether or extraction of the sample in water by a mixture of diethyl ether and petroleum ether in order to reduce the extraction of non-fatty components. Extraction of the dried material with petroleum ether is commonly used for fat determination in meat products, cereals and other low-sugar foods that are dry or easily dried. The extraction may be carried out in the cold (Soxhlet) or hot (Bolton, Goldfisch and Bailey-Walker extractors). Direct extraction of the dry material may give low results as the protein matrix has to be destroyed by acid or alkali.

The Rose-Gottlieb method is used for foods containing protein and sugar such as milk and milk products; the protein is dissolved by ammonia and not by acid (which acts on sugars yielding ether-soluble products). The method depends upon extraction from an ethanolic ammonia mixture by mixed diethyl and petroleum ethers. The ethanolic ammonia stabilizes the protein present. The same solvents are used in the Schmid-Bondzynski-Ratzlaff method, but protein is first decomposed by hydrochloric acid. The method is used for materials such as cheese but an undue amount of charring occurs in samples high in sugar. The Weibull-Stoldt method involves digestion of the sample in dilute acid, filtering and drying the fat together with any other material retained by the filter paper, completion being by extraction of the paper in a Soxhlet apparatus with petroleum ether. The method is the basis of that of the OICC for estimation of fat in chocolate and was found preferable to the Schmid-Bondzynski-Ratzlaff and Rose-Gottlieb methods for the determination of fat in baby food.

The fatty material in food does not consist only of fatty acid triglycerides, although these normally form by far the larger part. Small quantities of mono- and diglycerides may be present either naturally or as added emulsifiers. Many of the emulsifiers used in food are partly or wholly soluble in the solvents used to extract fat from samples. Sterols (such as cholesterol and beta-sitosterol) and phospholipids and their derivatives (such as lecithin, phosphatidyl inositol and other inositides) and phosphatidyl derivatives of amino-compounds (such as serine and ethanolamine) are also partly or wholly extractable under the experimental conditions of some commonly used methods. In most cases the error so introduced is not large as the proportion of fatty

material other than triglycerides is very low. Exceptions include egg yolk, the lipids of which contain only a little over 60% of triglycerides, the remainder being mainly lecithin, cephalin and cholesterol. Triglycerides may not be completely extracted by hexane, hot isopropanol or ethylene dichloride. Methods of fat determination have been received by Hannant (1) and Carter (2). There is a useful discussion in Pearson (3), pp 20-23.

CRUDE FAT

PRINCIPLE

Crude fat can be determined by extracting the dried ground food material with anhydrous ethyl ether or petroleum ether (BP 40°-60°C) in a continuous extraction apparatus of the Soxhlet type. The solvent is then removed from the extract by evaporation and the residue is weighed and reported as fat.

APPARATUS

1. Soxhlet extraction apparatus.

2. Extraction thimbles (Whatman cellulose, single thickness, 30mmX100mm).

3. Filter paper. (Whatman No. 42).

4. Desiccator.

5. Evaporating dish.

6. Water-bath.

REAGENTS

1. Ethyl ether, anhydrous.

PROCEDURE

Accurately weigh 3-4 g of sample (in a fine form) into a thimble lined with a circle of filter paper. Place thimble and contents into a 50 ml beaker and dry in a mechanical convection oven for 6 hours at 100-102°C or for 1-1/2 hours at 125°C. (Note: the sample must be free of moisture, otherwise some water-soluble material will be extracted and reported as fat. However, it is important to avoid excessive drying of samples, and to dry samples at 125°C or below to prevent possible oxidation of fat.)

Transfer thimble and contents to extraction apparatus. Rinse beaker several times with ethyl ether, adding rinsing to the apparatus. Extract the sample contained in the thimble with ethyl ether in a Soxhlet extraction apparatus for 6-8 hours at a condensation rate of at least 3-6 drops per second. At the completion of the extraction, transfer the fat extract from the extraction flask into a pre-weighed evaporating dish with several rinsings of ethyl ether. Place the evaporating dish in a fume hood and with the fan on, evaporate off the ethyl ether until no odour of it is detectable.

Dry the dish and contents in a mechanical convection oven for 30 minutes at 100°C. Remove from the oven, cool in a desiccator and weigh dish plus contents.

CALCULATION

$$\text{Crude fat (ether extract) \%} = \frac{(W_2 - W_1) \times 100}{S}$$

Where: W_1 = weight of empty evaporating dish

W_2 = weight of evaporating dish + contents after drying

S = sample weight in g.

REFERENCES

Official Methods of Analysis of the AOAC, 1984, 7.060-.062.

Stubbs, G. and More, A., 1919, Analyst 44, 125.

FAT
(Weibull-Stoldt Method)

PRINCIPLE

The sample is boiled with hydrochloric acid, filtered and the filter washed, dried and extracted into a tared flask with petroleum ether. The solvent is evaporated and the residue weighed. A blank should be run for the most accurate work.

APPARATUS

1. 250 ml conical flask.

2. Soxhlet extractor or similar.

REAGENTS

1. Petroleum ether, boiling range 40-60°C.

2. Hydrochloric acid, 33% v/v. Dilute 100 ml of concentrated hydrochloric acid with 200 ml of water.

PROCEDURE

Accurately weigh an amount of the sample preferably containing more than 0.1 g of fat, but preferably not more than 3 g of dry matter, into a 250 ml conical flask. Add 50 ml of dilute hydrochloric acid and a few glass beads and cover the flask with a watchglass. Boil for an hour, maintaining the volume by the addition of water. Add 150 ml of hot water, about 1 g of diatomaceous earth (e.g. "Hyflo-supercel") or about 100 cm^2 of filter paper torn in small pieces. Filter through a wetted fluted 15 cm Whatman 540 paper or equivalent (used double if preferred). A glass filter may be used to advantage. Wash the flask and watchglass thoroughly with hot water and pass the washings through the filter. Dry the flask and watchglass in the oven. Wash the filter with hot water until the filtrate is acid-free (test with blue litmus paper). Dry the filter paper on a watchglass at 120°C (60°C overnight if pieces of filter paper were added).

Use tongs to place the filter paper in a thimble. Transfer the thimble to a Soxhlet extractor. The flask may contain a few anti-bumping granules, which must have been included when the flask was weighed. Rinse the conical flask, watchglass and tongs with petroleum ether and add the rinsing to the Soxhlet and add sufficient extra solvent to bring the total quantity of solvent to one and a half to two times the capacity of the extractor. Heat at such a rate that at least 420 ml of petroleum ether has cycled through the extractor in 4 hours. Remove the flask and evaporate off the solvent using an empty Soxhlet or solvent-recovery unit for the bulk and completing the evaporation on a water-bath, taking care that heating is not so abrupt as to cause loss by vigorous boiling or spattering. Finally dry in the oven one hour at 102 $\pm$ 2°C. Dry to constant weight (successive weighings do not differ by more than 1 mg). Extract the paper in the Soxhlet for one hour with fresh solvent using a second weighed flask to check that extraction is complete. If the check residue exceeds 2 mg continue extraction until it is complete.

CALCULATION

$$\% \text{ fat} = \frac{\text{weight of residue}}{\text{weight of sample taken}} \times 100$$

If a second extraction provided a weighable residue the total weight of residue is used for the calculation.

REFERENCE

ISO 1443-1973.

TOTAL LIPIDS

PRINCIPLE

Total lipids comprise the total amount of fats (free and bound) and fat soluble substances, expressed in percent by weight obtained after acid hydrolysis of the sample, followed by extraction with hexane.

Lipids in protein and carbohydrate complexes are released by hydrochloric acid treatment, in the presence of ethanol and formic acid. Ethyl formate (which is produced in situ) dissolves the lipids and both are then extracted by hexane. They are determined gravimetrically after evaporating the solvent. The method is applicable to cereals, cereal products (including milled and baked goods) and animal feeds.

APPARATUS

1. Analytical balance, accurate to 0.1 mg. Range to 200 g.

2. Grinding mill.

3. Hand sieve, 0.400 mm.

4. Electric water-bath 75°C ± 1.

5. Extractor flask described in the figure with condenser (standard taper joints 29/32).

6. Round flask, 250 ml with ground glass joint.

7. Distillation apparatus with electric heater (rotary evaporator under vacuum preferably).

8. Magnetic stirrer with a Teflon-coated magnet.

9. Nitrogen gas.

(Note: A 160 ml extractor flask with standard taper joint 24/29 may be preferred for products with a lipids content exceeding 3%. Use 4 g of sample and half of the reagent volumes given in the method).

REAGENTS

1. Ethanol 95%.

2. Hydrochloric acid - water (50 + 20 v/v).

3. Formic acid 99%.

4. Hexane, BR 68-70°C (less than 0.001g residue per 100 ml).

PROCEDURE

The particle size of the sample should be such that less than 5% residue remains on a 0.4 mm sieve. Grind if necessary. Mix the ground sample to obtain maximum homogeneity.

Weigh 8 g of the sample into the extractor flask (with magnetic stirrer) and spread the sample out on the bottom of the flask. Add 8 ml ethanol and place the flask on the stirrer in order to obtain a homogeneous slurry as far as possible. Add 8 ml of formic acid and 12 ml of hydrochloric acid, then homogenize with the stirrer. Place the extractor flask (with a condenser) in the water-bath for 15 min, stirring the suspension by swirling from time to time. Remove the

condenser, cool the flask and place it on the magnetic stirrer. Add 20 ml ethanol and stir the mixture, then add 50 ml hexane, stir at maximum speed for 5 min. Decant the two phases. Pour the hexane into the round flask and the aqueous phase remaining into the side arm of the extractor flask. Rinse the neck of the extractor flask with a few drops of hexane. Introduce another 30 ml of hexane, stir the mixture 5 min. Repeat the extraction twice.

Evaporate the solvent preferably under reduced pressure using a rotary evaporator. Immediately after evaporation direct a stream of nitrogen for 10 min onto the surface of the lipids contained in the round flask. Weigh the extracted lipids.

CALCULATION

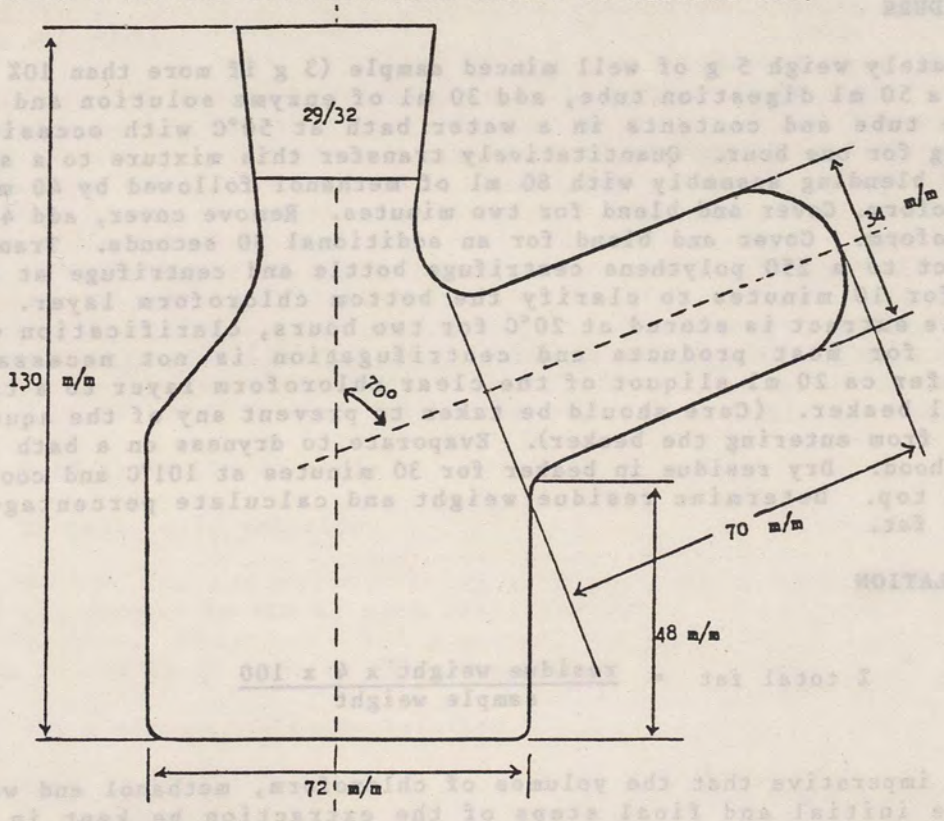
The total lipids content of the moist matter (mm) is calculated on the following basis:

$$\text{Lipids content (\% mm)} = \frac{\text{lipids (g)} \times 100}{\text{weight of sample (g)}}$$

The reduction to dry matter basis (dm):

$$\text{Lipids content (\% dm)} = \frac{\text{lipids (g)} \times 100}{\text{weight of dry matter (g)}}$$

The difference between duplicate determinations should not exceed 2%, otherwise a further determination should be carried out.



Extractor flask: Total volume - 317 ml; Volume of the side tube - 51 ml.

Available from Ets. Bercauverre S.A., 3 rue Rollin, 75005 Paris, France.

TOTAL FAT
(Chloroform-Methanol Method)

PRINCIPLE

The sample is digested using an enzyme and the released fat is extracted into chloroform-methanol. The fat is weighed after evaporation of the extracting solvent.

APPARATUS

1. Blender, semi-micro.
2. Digestion tube, 50 ml.
3. Water bath.
4. Centrifuge.

REAGENTS

1. Methanol.
2. Chloroform.
3. Enzyme solution: 2% Taka-diastase in 0.5 M sodium acetate: (Prepare as needed) Mix 25 ml of Parke-Davis Taka-diastase digestant with 200 ml of 2.5 M sodium acetate solution in a 1 L volumetric flask, dilute to volume and mix.

PROCEDURE

Accurately weigh 5 g of well minced sample (3 g if more than 10% fat) into a 50 ml digestion tube, add 30 ml of enzyme solution and mix. Place tube and contents in a water bath at 50°C with occasional mixing for one hour. Quantitatively transfer this mixture to a semi-micro blending assembly with 80 ml of methanol followed by 40 ml of chloroform. Cover and blend for two minutes. Remove cover, add 40 ml chloroform. Cover and blend for an additional 30 seconds. Transfer extract to a 250 polythene centrifuge bottle and centrifuge at 5000 rpm for 10 minutes to clarify the bottom chloroform layer. (If sample extract is stored at 20°C for two hours, clarification will occur for most products and centrifugation is not necessary). Transfer ca 20 ml aliquot of the clear chloroform layer to a tared 100 ml beaker. (Care should be taken to prevent any of the aqueous phase from entering the beaker). Evaporate to dryness on a bath in a fume hood. Dry residue in beaker for 30 minutes at 101°C and cool on bench top. Determine residue weight and calculate percentage of total fat.

CALCULATION

$$\% \text{ total fat} = \frac{\text{residue weight} \times 4 \times 100}{\text{sample weight}}$$

It is imperative that the volumes of chloroform, methanol and water in the initial and final steps of the extraction be kept in the proportions 1 : 2 : 0.8 and 2 : 2 : 1.8 respectively. This is necessary to ensure a quantitative extraction of total fat in the initial step and to prevent contamination of the chloroform layer with methanol and water in the final biphasic step.

REFERENCES

Official Methods of Analysis of the AOAC, 1984, 43.275-.277.

Bligh, E.G. & Dyer, W.J., 1959, Canadian Journal of Biochemistry and Physiology 37, 911.

8.3 Protein

The crude protein content of foods is traditionally determined by the organic nitrogen content using the Kjeldahl method. This is still an acceptable procedure and has the advantage of not requiring extensive equipment or sophisticated instrumentation. A table of calculation factors to convert organic nitrogen to crude protein is given below.

Conversion Factor Table

Food	Factor
Cereals	
Wheat whole meal or flour	5.83
Wheat flour (low or medium extraction)	5.70
Macaroni, spaghetti, wheat pastes	5.70
Wheat bran	6.31
Rice (all products)	5.95
Rye (all products)	5.83
Barley (all products)	5.83
Oats (all products)	5.83
Pulses, Nuts and Seeds	
Groundnuts	5.46
Soya bean (all products)	5.71
Almonds	5.18
Brazil nuts	5.46
Coconut	5.30
Chestnuts	5.30
Seeds (sesame, safflower, sunflower)	5.30
Other seeds	5.30
Milk and Dairy Products	
Milk (all-fresh or dry)	6.38
Cheeses (all)	6.38
Butter (and margarines)	6.38
Other Foods	6.25

CRUDE PROTEIN
(Kjeldahl Macro Method)

PRINCIPLE

The sample is digested in sulphuric acid in the presence of a catalyst. The nitrogen from protein and some other constituents is converted to ammonium sulphate. The ammonia is distilled into standard acid after the digest has been made alkaline. The percentage of nitrogen is calculated and the result converted to "crude protein" by multiplication by a factor (usually 6.25).

APPARATUS

1. Kjeldahl flasks, borosilicate glass, 300, 500 or 800 ml.

2. Distillation unit (see figure).

REAGENTS

1. Sulphuric acid. Very high purity is not essential but the nitrogen should be less than 0.0005%.

2. Catalyst: many combinations of catalyst have been used, for example 10 g potassium sulphate and 0.5 g mercuric oxide per digestion. If mercury is used, sodium sulphide or thiosulphate must be added later to decompose mercury ammonium compounds. The use of mercury should be avoided where possible. 1000 g potassium sulphate, 30 g copper sulphate pentahydrate and 30 g titanium dioxide ground together or mixed in a ball mill or powder mixer gives an effective substitute. Alternatively, ready prepared tablets may be obtained from suppliers.

3. Saturated sodium hydroxide. This should be prepared in a stout polythene bucket or other suitable container. (The solution attacks glass which should therefore not be used). Place about 3 litres of water (tap water may be used) in the bucket and add with constant stirring 2.5 kg of technical grade sodium hydroxide. After this has dissolved add water until the 5-litre mark is reached. (The bucket may be calibrated at this level before use, if desired). The solution should be left at least 24 hours for sodium carbonate to settle, and the supernatant used for the determination. Eye protection should be worn during the preparation of the caustic solution. It is convenient to carry out the operation in a sink half-full of water to cool the solution during the initial stages.

4. 0.1N hydrochloric acid.

5. 2% boric acid solution.

6. Methyl red indicator. 0.1 g is dissolved in 18.6 ml of 0.02N NaOH and diluted to 250 ml with distilled water. Screened methyl red may be used. Dissolve 0.016 g methyl red and 0.083 g bromocresol green in 100 ml of neutral 96% ethanol.

7. 0.1 N sodium hydroxide solution.

PROCEDURE

Place about 1 g of sample, accurately weighed, in the Kjeldahl digestion flask. Moist samples such as sausages are conveniently weighed on a tared filter paper, on a watch glass and put in the flask wrapped in the paper. The weight of sample taken should be such as to neutralize about 20 ml 0.1 N acid (i.e., contain about 0.03 g of nitrogen). Add 25 ml of sulphuric acid and 10 g of

catalyst and digest in a fume hood, slowly at first to prevent undue frothing. Continue to digest for at least 45 minutes after the digest has become a clear pale green. Only 30 - 40 minutes may be used for the total digestion in routine control where speed is more important than accuracy. Leave until completely cool, and rapidly add 100 - 200 ml water. Mix and transfer to the distillation flask, rinse the digestion flask 2 or 3 times and add the rinsings to the bulk.

Add 80 - 85 ml of saturated sodium hydroxide solution from a measuring cylinder so that ammonia is not lost. If after shaking, the digest does not turn blue due to copper hydroxide, it indicates that insufficient alkali had been added. Distil into 25 ml of 0.1 N hydrochloric acid containing a few drops of methyl red indicator. Alternatively distil into 50 ml of 2% boric acid containing screened methyl red. The boric acid is neutral to this indicator and the alkaline ammonium borate formed is titrated directly with a 0.1 N HCl which is then the only standard solution required. The exact strength of the boric acid is not important. If insufficient standard acid has been added it is permissible to pipette a further quantity into the flask provided this is done as soon as the solution shows signs of becoming alkaline. The acid may tend to suck back into the condenser at the beginning and end of the distillation. This is easily avoided by using an Allihn condenser and by adjusting the tube on the end of the condenser so that it is only just below the level of the acid, and the suction is broken by the liquid rising up the condenser. Distil until the contents of the flask "bump". Titrate the excess acid with 0.1N NaOH.

(Note: If rubber stoppers are used, the flask is inclined and sodium hydroxide solution carefully added down the side of the neck, so as to form a layer under the diluted sulphuric acid. The stoppers on the splashhead may then be wetted with a few drops of water and the apparatus connected together. Not until then is the flask shaken so as to mix the contents. Distillation is commenced immediately).

CALCULATION

$$\% \text{ nitrogen in sample} = 14 \times \frac{V}{1000} \times 0.1 \times \frac{W}{100}$$

Where: V = ml of 0.1 N acid added - ml of 0.1 N NaOH used to neutralize the ammonia nitrogen).

W = g of sample.

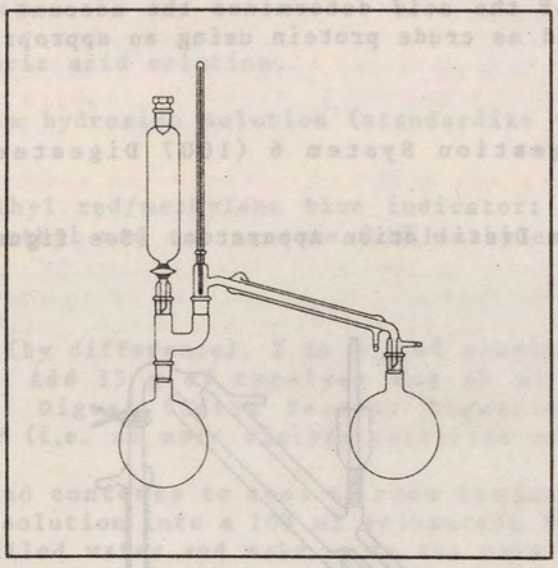
INTERPRETATION

N x 6.25 = crude protein. 6.25 is a general factor suitable for products in which the proportions of specific proteins is not well defined. For factors for selected foods see the table under 8.3.

Ammonia and ammonium salts and urea would be included but are not normally present in significant amounts. Nitrates, nitrites and nitroso compounds are not converted to ammonium salts by this method. Nitrogen from amino-acids and such additives or constituents as glutamate, guanylate (flavour-enhancers), creatine and creatinine (from meat extracts) is also determined by the method. Crude protein calculated from the percentage of nitrogen will be erroneously high in samples containing compounds of higher average percentage nitrogen than protein. Specific methods for glutamate, creatine and creatinine should be used.

The sodium or potassium sulphate is added to raise the temperature and thus speed the digestion. In most methods the volume of acid added initially is 2-

1/2 times the weight of catalyst. In fact, it is the ratio towards and at the end of the digestion which is important and should be about 1:1, more acid possibly resulting in incomplete digestion and less acid in loss of nitrogen. Assuming that fat requires 10 ml per g for digestion and carbohydrate 4 ml per g, the amount of acid to be added can be approximately estimated. Digests that go quite solid on cooling should be discarded.



Distillation Apparatus

REFERENCES

Official Methods of Analysis of the AOAC, 1984, 2.055-.057.

Lakin, A.L., 1978, Developments in Food Analysis Techniques-1. King, R.D. (Ed.), Applied Science.

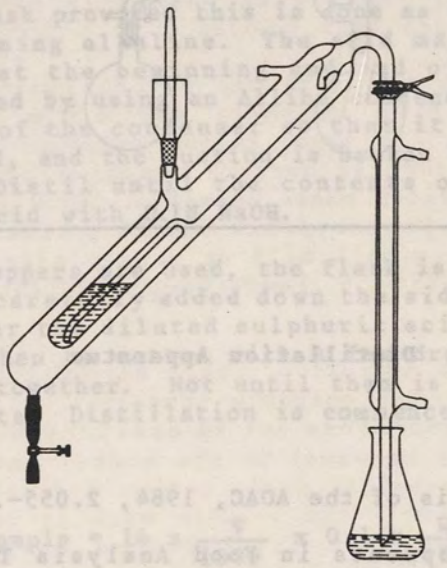
CRUDE PROTEIN
(Semi-Micro Method)

PRINCIPLE

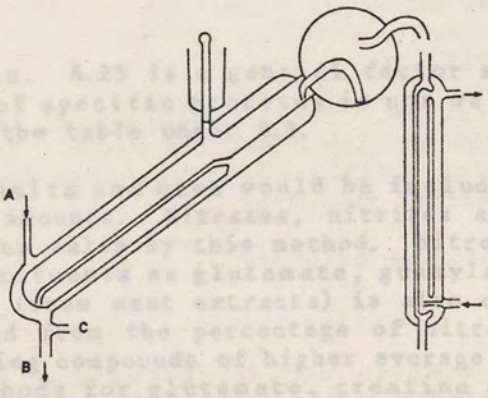
The sample protein is digested using sulphuric acid and copper as a catalyst. Sodium sulphate is added to raise the boiling point. The resultant ammonia is released by adding alkali and is steam distilled into a standard acid solution. A residual titration of the acid determines the account of ammonia distilled. This is then calculated as crude protein using an appropriate factor.

APPARATUS

1. Tecator Digestion System 6 (1007 Digester) and tubes or equivalent.
2. Markham Steam Distillation Apparatus. (See figure).
3. Burettes.
4. Pipettes.



Markham semi-micro Kjeldahl distillation apparatus
(by courtesy of Quickfit and Quartz, Ltd.).



Yuen and Pollard's modification of the Markham semi-micro Kjeldahl distillation apparatus, incorporating splash-head.
A = steam inlet; B = water outlet; C = safety outlet

REAGENTS

1. Catalyst - 1 part copper sulphate plus 10 parts sodium sulphate. Grind to powder in a mortar.

2. Concentrated sulphuric acid.

3. Sodium hydroxide solution (40%): Dissolve 40 g NaOH in water and make to 100 ml.

4. 0.1 N sulphuric acid solution.

5. 0.02 N sodium hydroxide solution (standardize vs the 0.1 N acid solution).

6. Screened methyl red/methylene blue indicator: Mix equal parts of aqueous 0.2% methyl red and aqueous 0.1% methylene blue.

PROCEDURE

Accurately weigh (by difference), 2 to 3 g of sample into the Tecator digestion tube. Add 15 g of catalyst and 35 ml of concentrated sulphuric acid. Digest (using Tecator Digestion System) until solution is clear (i.e. no more visible particles of sample).

Allow the tube and contents to cool to room temperature. Transfer the clear digest solution into a 100 ml volumetric flask with several rinsings of distilled water and make up to the mark when solution has cooled (Precaution: Heat is liberated in the process, therefore cool flask in cold water bath). Pipette 5 ml and transfer to the steamed-out Markham Distillation apparatus through the funnel of the apparatus.

Stopper funnel, add 5 ml of 40% sodium hydroxide to the funnel. Attach a 100 ml distillation receiving flask containing 5 ml of 0.1 N acid with about 6-8 drops of screened mixed indicator added. (Make sure the tip of the receiver is below the surface of the acid in the flask.) When steam reaches the condenser, slowly run down the 40% sodium hydroxide solution until the digest turns brown. Distil for 5 minutes.

Lower the distillation flask and rinse down the condenser with distilled water. Titrate the distillate with 0.02N sodium hydroxide solution to a permanent green end-point. Carry out a blank titration by titrating 5 ml of 0.1 N acid with 0.02N base.

CALCULATION

$$\text{Total Nitrogen \%} = \frac{(B - S) \times 1.4007 \times N}{\text{Sample Weight g}} \times 20$$

Where: B = ml of NaOH solution used for blank
S = ml of NaOH solution used for sample
N = Normality of NaOH

1.4007 = meq. wt. of nitrogen (includes factors of 100 for %)

20 = Dilution factor (5 ml of digest used out of 100 ml)

Crude Protein % = total nitrogen % x protein conversion factor (see table in 8.3).

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Markham, R., 1942, Biochemical Journal 36, 790.

Hoskins, J.L., 1944, Analyst 69, 271.

Yuen, S.H. and Pollard, A.G., 1953, Journal of the Science of Food and Agriculture 4, 490.

8.4 Ash

The ash of a food represents its inorganic residue after the organic carbonaceous portion and other volatiles have been oxidized and evaporated away. Ash often can serve as a measure of adulteration of a food. For example, a higher ash level than expected could indicate addition of adulterants having a higher inorganic level than the food. Conversely, a low ash finding could mean dilution of the food with material having a low inorganic level.

Some foods have unique ashing problems, many of which were discussed in the section on metals analysis. For example, for high fat foods the fat should be carefully smoked off without ignition, by using a heat lamp or burner, or at the lip of an open muffle furnace.

For foods which do not ash satisfactorily using the general method, break up the pieces with a thick platinum wire. Addition of a few drops of water to the cooled dish, evaporation and re-ashing may be enough to obtain a white ash from samples such as flour. If this is also inadequate, cool the dish, add a few ml of water and filter through an ashless paper. Return the paper and contents to the dish, dry and re-ash. As a last resort, the dish may be cooled and a few drops of 10% nitric acid or 50% ammonium nitrate added, evaporated and ashing continued.

TOTAL ASH

PRINCIPLE

The ash of a foodstuff is the inorganic residue remaining after the foodstuff is ignited until it is carbon free (i.e. after the organic matter has been burnt away), usually at a temperature not exceeding red heat. The ash obtained is not necessarily of exactly the same composition as the mineral matter present in the original food as there may be losses due to volatilization or some other interaction between constituents. The ash figure can be regarded as a general measure of quality and often is a useful indication of identity.

APPARATUS

1. Porcelain dish.
2. Drying oven.
3. Muffle furnace.

PROCEDURE

Weigh 5 g of the sample into a weighed porcelain dish. Dry at 100°C for 3-4 hrs in a mechanical convection oven. Remove the porcelain dish from the oven. Do an initial carbonization by placing dish over a bunsen flame. Heat gently until the contents turn black (for sugars and sugar products add a few drops of pure olive oil and heat until swelling stops).

Transfer the dish and contents to a muffle furnace and ignite at 500 to 600°C until free from carbon (residue appears greyish-white) (about 8 hr). Remove from muffle oven and moisten this first ash with a few drops of water. (This is to expose bits of unashed carbon).

Re-dry in oven at 100°C for 3-4 hrs, and re-ash at 500-600°C for another hour. Remove from muffle furnace, allow to cool for a moment, place in a desiccator until cool, and weigh. Calculate and express results as % Ash.

CALCULATION

$$\text{Ash \%} = \frac{(B-C) \times 100}{A}$$

Where: A = sample weight in g.

B = wt. in g of dish and contents after drying.

C = wt. in g of empty dish.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 14.006, as well as other references under other commodity headings.

8.5 Other Constituents

The previously discussed food constituents (moisture, fat, protein and ash) can be considered to be 'natural' or non-added. Crude fibre is also in this category. It generally represents that portion of a food which is not used by the body. It can also indicate adulteration by high-fibre materials such as wood saw-dust.

Other constituents such as salt (sodium chloride) and starch may be a normal part of a food, but are most often added. An example is cereal (starch) products added to processed meat foods as a filler, or salt present as a seasoning or a preservative.

CRUDE FIBRE

PRINCIPLE

Crude fibre is the organic residue left after the defatted material has been treated with boiling dilute sulphuric solution, boiling dilute sodium hydroxide solution, dilute hydrochloric acid, alcohol and ether.

APPARATUS

1. 1-litre conical flasks.

2. Buchner funnel and flask, funnel to take 11 cm papers, and vacuum pump.

3. Hot plate.

4. Silica or porcelain crucibles.

5. Muffle furnace.

REAGENTS

1. Petroleum ether.

2. Sulphuric acid, 0.255 N, standardized.

3. Sodium hydroxide solution, carbonate-free, 0.313 N, standardized.

4. Ethanol, 95%.

5. Diethyl ether.

6. 1% hydrochloric acid.

PROCEDURE

Accurately weigh 3 g of sample into a 1-litre conical flask. If the oil content is over 1%, add petroleum ether, swirl, leave to stand and carefully decant and repeat twice more, preferably leaving the last quantity of solvent in contact overnight, with a small watchglass over the mouth of the conical. Decant the solvent carefully avoiding loss of particles of fibre and warm gently to remove visible solvent. Alternatively extract the sample on the Soxhlet in a thimble or hardened paper that will not contribute fibre.

Add 200 ml of boiling 0.255 N acid and place the flask on a hot plate or over a Bunsen burner so as to return the solution to the boil as quickly as possible. Mark the glass at the liquid surface. Place a funnel of about 8 - 10 cm diameter in the mouth to diminish evaporation. As soon as the liquid boils, control the heating so that gentle ebullition is maintained and continue for 30 + 2 minutes. (If frothing is excessive, add a drop of antifoam.) Add boiling water to maintain the volume if necessary. Swirl occasionally to remove solids from the sides of the flask.

Prepare a Buchner flask and funnel connected via a trap to a vacuum pump. Place a Whatman 11-cm No. 52 paper or equivalent in the funnel, fill with hot water. At the end of the boiling period remove the flask from the heat, leave to settle a few moments and decant through the Buchner funnel, applying gentle suction such that the funnel is not permitted to empty completely until most of the flask

contents are transferred. If the solution is maintained well-mixed instead of decanted, the fibre particles may block the filter opposite the funnel holes. Increase suction as necessary. Rinse the conical flask with near-boiling water so as to transfer all trace of acid through the filter. Removal of all solid particles is not essential. (Since plastic washbottles soften when containing hot liquids, it may be preferred to use a glass one, blowing by mouth to provide the necessary pressure.) After rinsing the acid from the walls of the conical flask and draining into the funnel, wash the filter liberally with hot water and drain. Turn off the vacuum. Raise the edge of the paper with a spatula, and lay flat on the side of the funnel, the latter being in the neck of the flask.

Pour a measured 200 ml of near boiling 0.313 N base solution into a washbottle with a fine jet and use this solution to wash the residue on the paper into the flask, then wash any particles of fibre from the Buchner funnel into the flask. Pour any unused base solution into the flask, bring to the boil as quickly as possible and maintain gentle ebullition for 30 $\pm$ 2 minutes. Filter through an 11- or 12.5-cm rapid hardened paper (e.g. Whatman 541) in an ordinary conical filter funnel. As soon as the bulk of the fibre and solution are transferred, wash the filter paper with enough 1% hydrochloric acid to make the paper and contents acid. This is usually obvious by a change in colour, but check with an indicator paper at the funnel tip if necessary. Use water to transfer any remaining particles from the conical flask to the paper and wash the paper with water until acid-free, then wash with alcohol and diethyl ether until substantially all the water is removed. (This last stage must be carried out away from any naked flame). Leave the residue to air-dry and then remove and open the paper and carefully transfer the residue to a tared clean and incinerated crucible with the aid of a spatula. Take care not to include fibres from the filter paper but to include all residue. Dry in the oven, cool and weigh. Incinerate at 500°C, cool and weigh. The loss in weight represents the fibre content. The fibre residue from the test consists mainly of cellulose, with some lignin, but not all of the cellulose is determined.

CALCULATION

$$\% \text{ fibre} = \frac{\text{Loss in weight from incineration}}{\text{Weight of sample before defatting}} \times 100$$

INTERPRETATION

This is an empirical method and results depend on adherence to the exact conditions of test. The results vary with the particle size of the sample. The difficulty of reducing the fibrous part of the sample to as fine a state as desirable leads to error both from nonhomogeneity and diminished attack by the acid and alkali. The usefulness of the test often lies in confirming the absence of fibrous adulterants such as sawdust and the test may be reported as "fibre negligible" or "fibrous adulterants not detected" or even omitted from the report, as the important point is that the analyst has satisfied himself as to the genuineness of the sample. On the other hand, if it is necessary to use a quantitative result, as for a sample claiming to be wholemeal flour and low in fibre, then 3 or 4 determinations at least should be done. The sample should be very carefully mixed and even then there is inherent uncertainty in the result.

REFERENCES

Official Methods of Analysis of the AOAC, 1984, 7.066-.070.

ISO 5498-1981. Agricultural Food Products - Determination of Crude Fibre Content - General Method.

The Feeding Stuffs (Sampling and Analysis) Regulations, 1982, S.I. 1982 No. 1144 HMSO.

SALT
(Volhard Method)

PRINCIPLE

To determine salt, an analysis is made for total chlorides and the result is expressed as sodium chloride. The method involves the digestion of the sample with concentrated nitric acid and oxidation of any remaining organic matter with potassium permanganate. The dissolved chlorides in the sample are precipitated with excess silver nitrate as silver chloride, and the excess silver ions backtitrated with thiocyanate using ferric alum as the indicator. When all the excess silver ions have reacted with thiocyanate, any slight excess of thiocycnate will react with ferric alum to form a red-coloured complex (ferric thiocyanate) at the end-point. An excess of thiocyanate may react with the precipitated silver chloride, since the solubility product of silver thiocyanate is 0.01 that of silver chloride. The addition of nitrobenzene, diethyl ether or acetone overcomes this difficulty by coating the precipitated silver chloride and thereby withdrawing it from reaction with the thiocyanate solution. Nitrobenzene is toxic and its use is best avoided.

APPARATUS

1. Pipettes and burettes.

2. Laboratory glassware.

REAGENTS

1. Ferric alum indicator:saturated aqueous solution of reagent grade ferric ammonium sulphate ($12H_2O$).

2. Silver nitrate solution, 0.20 N - dissolve 17.04 g of silver nitrate, (previously dried at 110°C) in distilled water and dilute to 1 litre. Standardize (using excess silver nitrate solution) against 0.10 N sodium chloride (5.845 g per litre).

3. Potassium thiocyanate solution (0.100N) - dissolve 9.72 g of reagent grade potassium thiocyanate in distilled water, and dilute to one litre.

4. Concentrated nitric acid.

5. Nitric acid (1+1).

6. Nitrobenzene.

7. Potassium permanganate solution (5%).

PROCEDURE

Accurately weigh appropriate amount (about 2-3 g) of sample into a 300 ml Erlenmeyer flask. (For shrimp paste or other highly salted foods, weigh about 5 g into 250 ml volumetric flask and digest for about one hour in water bath to dissolve the salt. Filter through No. 1 filter paper. Take appropriate amount of this filtrate) (e.g. for shrimp paste, take 10 ml). Add 25 ml of 0.100N silver nitrate solution, swirl flask until sample and solution are in intimate contact. Add 15 ml of concentrated nitric acid. (The silver nitrate solution must be added first, followed by the concentrated nitric acid. The order of addition ensures complete precipitation of the chlorides. If nitric acid is added first, loss of chloride by volatilization as hydrogen chloride could occur, since hydrogen chloride has a far greater vapour pressure than nitric acid.)

Boil in a fume hood until dissolved and add potassium permanganate solution (5%) until colour disappears and the solution becomes colourless. (Should too much potassium permanganate be accidently added, colour removal can be effected by the addition of small quantities of sugar). Add about 15 to 25 ml of distilled water, boil for 5 mins. Cool and dilute to about 150 ml with distilled water. Add about 1 ml nitrobenzene, 2 ml of ferric alum indicator and shake vigorously to coagulate the precipitated silver chloride. Titrate the excess silver nitrate with 0.100 N potassium thiocyanate solution to a permanent light brown end-point.

Carry out a blank titration containing 25.0 ml of 0.100N silver nitrate solution, 2 ml ferric alum indicator and 15 ml of (1+1) nitric acid with 0.100N potassium thiocyanate.

CALCULATION

$$\text{Salt (as \% NaCl)} = \frac{(B-T)(N)(5.85)}{S}$$

Where:

B = ml of potassium thiocyanate solution used for titration of blank.

T = ml of potassium thiocyanate solution used for sample titration.

N = Normality of potassium thiocyanate.

S = Sample weight (in g).

5.85 = milliequivalent weight of sodium chloride (including the factor of 100%).

For highly salted foods requiring dilution, multiply the above calculation by 250/ml aliquot taken for analysis.

REFERENCE

Official Methods of Analysis of the AOAC, 1984, 32.033, and other references under other commodity headings.

STARCH

PRINCIPLE

Cereal is often added to meat products as a binder. This method for the estimation of starch in meat products depends on the solution of the protein plus other nitrogenous, fatty and salt components of meat in an alcoholic solution of potassium hydroxide. The fat is saponified and the protein hydrolysed. Spices, cellulose and starch remain as a sediment. The cereal starch is then dissolved in hydrochloric acid, re-precipitated with ethanol and determined gravimetrically.

APPARATUS

1. Centrifuge and tubes.

2. Steam bath.

REAGENTS

1. 95% ethanol.

2. 8% alcoholic potassium hydroxide solution : 40 g KOH dissolved in 300 ml of 95% ethanol and diluted to 500 ml with 95% ethanol.

3. Hydrochloric acid-water (1+1).

PROCEDURE

Add about 50 ml of 8% alcoholic KOH solution to 10.0 g of sample and digest on a steam bath for 20 minutes with occasional stirring. Transfer to centrifuge tubes. Ensure tubes are balanced and centrifuge for 5 minutes at 2000 rpm.

Decant and discard the supernatant. Wash the sediment with 25 ml of 95% ethanol, stirring thoroughly. Centrifuge for 5 minutes at 2000 rpm. Decant and discard supernatant. Add 50 ml of HCl (1+1). Mix thoroughly to dissolve the cereal starch.

Transfer 25 ml of clear solution to a 150 ml beaker containing 75 ml of 95% ethanol. Mix well and let stand overnight. (Cover beaker with a watch glass to prevent extraneous matter from entering.) Filter through a weighed dried filter paper that has been pre-washed with 95% ethanol. Wash filter paper and contents with two 25 ml portions of 95% ethanol. Dry for 30 minutes (or until constant weight) at 75°C and weigh.

CALCULATION

$$\text{Starch, \%} = \frac{(100)(A-B)(2)}{S}$$

Where: A = g of filter paper + contents after drying.

B = g of dried filter paper.

S = Sample weight in g.

2 = Correction factor.

8.6 TEXT REFERENCES

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3. EGAN, H., KIRK, R.S. & SAWYER, R. 1981. Pearson's Chemical Analysis of Foods 8th Ed., Churchill, Livingstone.

Further Reading

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Composition of Foods, Agriculture Handbook No. 8 (Revisions of) US Dept. of Agriculture, Washington.

APPENDIX

Abreviations Used in the Manual

Units of Measure

A	=	absorbance
g	=	gram
kg	=	kilogram (10^{3} g)
mg	=	milligram (10^{-3} g)
µg	=	microgram (10^{-6} g)
ng	=	nanogram (10^{-9} g)
L	=	litre
ml	=	millilitre (10^{-3} L)
µl	=	microlitre (10^{-6} L)
m	=	metre
cm	=	centimetre (10^{-2} m)
mm	=	millimetre (10^{-3} m)
in	=	inch(es) (25 mm)
hr	=	hour(s)
min	=	minute(s)
sec	=	second(s)
rpm	=	revolutions per minute
°C	=	degrees Celsius (centigrade)
R_f	=	ration of: $\frac{\text{distance spot moved}}{\text{distance solvent moved}}$

Descriptive Units

AR	=	analytical reagent (grade)
BR	=	boiling range
EDTA	=	ethylene diamine tetracetic acid
G/G	=	ground glass
IU	=	International Units
MW	=	molecular weight
%	=	parts per hundred (percent)
ppm	=	parts per million
ppb	=	parts per billion
max	=	maximum
min	=	minimum
ave	=	average
M	=	molar
N	=	normal
ID	=	interior diameter
OD	=	outside diameter

m/m	=	mass in mass
m/v	=	mass in volume
v/v	=	volume in volume

Analytical Techniques

AAS	=	atomic absorption spectrophotometry
GLC	=	gas-liquid chromatography
HPLC	=	high performance liquid chromatography
PC	=	paper chromatography
TLC	=	thin-layer chromatography
RI	=	refractive index
SG	=	specific gravity
IR	=	infrared
UV	=	ultraviolet
vis	=	visible

Organizations and Agencies

AACC	=	American Association of Cereal Chemists
AOAC	=	Association of Official Analytical Chemists
AOCS	=	American Oil Chemists Society
BP	=	British Pharmacopoeia
BS	=	British Standards
CAC	=	Codex Alimentarius Commission
EEC	=	European Economic Community
ICC	=	International Association for Cereal Science and Technology
ICUMSA	=	International Commission for Uniform Methods of Sugar Analysis
ISO	=	International Standardization Organization
IUPAC	=	International Union of Pure and Applied Chemistry
OIV	=	Office International de la Vigne et du Vin

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2. Report of the joint FAO/WHO/UNEP conference on mycotoxins, 1977 (E* F* S*)
3. Report of the joint FAO/WHO expert consultation on the rôle of dietary fats and oils in human nutrition, 1977 (E* F* S*)
4. JECFA specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers, 1978 (E*)
5. Guide to JECFA specifications, 1978 (E* F*)

5 Rev. 1. Bibliography of food consumption surveys, 1984 (E*)

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E — English
F — French
S — Spanish

* Available
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