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FAO JECFA Monographs 1

COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

All specifications monographs from the
1st to the 65th meeting (1956–2005)

Volume 4

Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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FAO Food and Nutrition Paper 52, "Compendium of Food Additive Specifications" (FNP 52) was first published in 1992. It combined all of the food additive specifications prepared by JECFA from its first meeting in 1956 to its thirty-seventh meeting in 1990. A companion publication, FAO Food and Nutrition Paper 5, "Guide to Specifications" (FNP 5) contained the analytical methods, test procedures and laboratory solutions used in and referenced by JECFA food additive specifications. It was first published in 1978 and revised in 1991.

The decision was made to prepare an updated second edition of FNP 52 to include all food additive specifications prepared by JECFA through its sixty-fifth meeting in 2005. It was further decided that an updated and revised version of FNP 5 would not be published separately as before, but would be incorporated into the FNP 52 Compendium second edition. The new Compendium of Food Additive Specifications is therefore now comprised of four volumes. The first three volumes are the food additive specifications themselves in alphabetical order. This fourth volume includes the revised and updated analytical methods, test procedures and laboratory solutions which first appeared in FNP 5, plus newer procedures and an expanded section on laboratory instrumentation.

The review, revision and updating of the material in FNP 5, to prepare Volume 4, was a long and arduous process. The Food and Agriculture Organization of the United Nations (FAO) wishes to acknowledge and thank the following experts who were instrumental in the preparation and finalization of this document: (Listed in alphabetical order)

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- Dr. Brian Whitehouse, Consultant, United Kingdom

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SPECIAL NOTE

The methods and analytical procedures described in this Compendium 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.

COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

INTRODUCTION

Introduction Contents

FOREWORD

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) was established following the recommendation of the first Joint FAO/WHO Conference on Food Additives that the two organizations collect and disseminate information on food additives (Joint FAO/WHO Conference on Food Additives, Report. FAO Nutrition Meetings Report Series, No. 11; WHO Technical Report Series, No. 107, 1956). JECFA was first convened in 1956, and has met annually, with a few exceptions, since that time.

As part of its work, JECFA establishes specifications of identity and purity for food additives. These were originally published in FAO Nutrition Meetings Reports Series (NMRS), the WHO Technical Report Series (TRS) or as FAO Food and Nutrition Papers (FNP). However many of these are now out of print, and the first edition of this Compendium was published in 1992 in order to consolidate all of the then current JECFA specifications into a single publication. Since 1992, separate Addenda to this Compendium have been published which contain both newly established specifications and revisions to earlier specifications. The original Compendium and the succeeding Addenda were all published in the FNP series as number 52. This new Combined Compendium replaces the earlier edition and incorporates all the additions and revisions made since 1992, up to and including those contained in FNP 52 Addendum 13. It is being published as the first document under a new publication series, the FAO JECFA Monographs.

Many additive specifications have been revised two or more times as new information or circumstances require. The specifications contained in this Combined Compendium are the most recent version or revision for each additive. This new Combined Compendium is in four volumes as follows:

- Volume 1 – Specifications for food additives A through D.
- Volume 2 – Specifications for food additives E through O.
- Volume 3 – Specifications for food additives P through Z.

- Volume 4 – Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications.

This Introduction is intended to replace and update the sections in the IPCS document *Principles for the Safety Assessment of Food Additives and Contaminants in Food* (Environmental Health Criteria 70 (EHC 70), WHO, 1987) that describe the purpose and function of specifications of identity and purity of food additives. It also incorporates the General Notices section of the *Guide to Specifications* originally published in Food and Nutrition Paper 5 (FNP 5), revision 2. The remainder of FNP 5 has been extensively revised and updated and is now included in Volume 4.

The term 'specifications' refers to the full set of individual specifications criteria for an additive. Specifications are regarded as one of the outputs of JECFA's risk assessment of additives. The term 'safety evaluation' is used to describe the output of JECFA's assessment of the toxicology and other safety data relating to a food additive.

As previously noted, the specifications published in this Combined Compendium represent the specifications current at the time of publication. These are, however, subject to future review and revision as part of JECFA's continuing work. New and revised specifications are published as soon as practicable after the JECFA meetings when these were considered, and JECFA specifications are also available online on the FAO website.

Specifications for flavouring agents are not included in this Compendium, excepting those few which have an additional technological function as a food additive (e.g. carrier solvent). Flavouring agent specifications will, however, still be available online in a searchable database at the FAO website.

PURPOSE AND FUNCTION OF SPECIFICATIONS OF IDENTITY AND PURITY OF FOOD ADDITIVES

General

The specifications of identity and purity established by JECFA are intended to ensure that the Committee's safety evaluations apply, with a high degree of confidence, to all food additives manufactured according to those specifications. In setting specifications, the Committee also takes into account the need to encourage good manufacturing practice and to maintain the quality of additives on the market, noting that these considerations may add to the assurance of safety that the Committee is seeking as part of its risk assessment function.

JECFA's specifications are also intended to set manufacturing standards for food additives traded in international commerce either as such, or incorporated in food products. In some cases, JECFA specifications will differ from specifications developed by other national and international organizations. JECFA's safety evaluations can, however, be taken to apply to additives complying with these other specifications, provided that the additives are of equal or higher purity than those required by the JECFA specifications, and provided that the test criteria in the JECFA specifications are met.

The setting of specifications of identity and purity is an essential part of the risk assessment of food additives, and current thinking emphasizes the need for the safety evaluation and the specifications to be seen as joint outputs of the risk assessment process. The safety evaluation of an additive should therefore always be read in conjunction with the specifications of identity and purity that describe the additive. The link between the two parts of the risk assessment process is also emphasized by the fact that the latest safety evaluation is quoted in each of the specifications, together with a reference to the JECFA meetings(s) at which the specifications were elaborated and the safety evaluation carried out.

Formulation of Specifications and Information Requirements

The formulation of satisfactory specifications requires detailed information to be made available to the Committee on the method of manufacture of the additive, including information on raw materials and on its chemical characterization. The Committee requires such information to be provided as part of the total data package whenever an additive is submitted for risk assessment, and all such information will be regarded as suitable for making publicly available unless requested otherwise and agreed by the Secretariat. Those submitting data for a JECFA evaluation are advised to consult existing specifications for further guidance, taking note also of the format of specifications described later in this introduction. They should also seek further advice as needed from the JECFA Secretariat.

Specifications may be revised where there is new information available on methods of manufacture or on the characteristics of the substance, or where changes or revisions in analytical methods are needed. Such changes may also prompt a review of the safety evaluation. Similarly, a review of the specifications may be needed if the safety evaluation is reconsidered.

Additives are mainly defined by a combination of (i) a description of their manufacture, (ii) a minimum requirement for the content of the principal functional component(s) of the additive, and (iii) maximum limits for undesirable impurities. The relative importance of these criteria, however, depends on the nature of the additive. Thus, for example, additives composed largely of single components are mainly defined in terms of their chemical purity,

whereas the definition of more complex materials, e.g. natural gums, relies more on a description of the raw materials and the method of manufacture.

In some cases, there may be insufficient information for the Committee to elaborate what it regards as fully acceptable specifications. The Committee may then decide to publish the incomplete specifications, but with a designation of 'Tentative'. In such cases the Committee will also state what additional information is required, and set a date by which this must be provided. The Committee will reconsider the specifications once the necessary information has been received, and if it considers that the information is sufficient, it will remove the 'Tentative' designation. If, however, the information is still deficient, or if no information has been provided by the due date, the specifications will be withdrawn.

JECFA specifications incorporate the methods of analysis that are to be used in testing the individual criteria. Information provided in submissions to JECFA should, therefore, always include details of the analytical methods to be used.

Stability and Fate of Additives in Food

Specifications are intended to apply to the additive as marketed. However, some food additives are designed to perform their function by interacting with components of food as in the case of flour improvers, or, for example by removing undesirable constituents, as in the case of antioxidants. Others may be subject to chemical degradation in food or may interact with food components, sometimes with the production of undesirable reaction products.

In order to ensure that test data are relevant to the way the additive is used in food, the Committee requires information on potential reactivity to be provided as part of submissions for the safety evaluation of additives. This information should include data on (a) the general chemical reactivity of the additive; (b) its stability during storage and reactions in model systems; and (c) the reactions of the additive in food systems. The Committee may also set specific limits on potential degradation products in those cases where the additive may be subject to degradation during storage.

JECFA SPECIFICATIONS AND THE CODEX SYSTEM

JECFA, CCFAC and the Risk Analysis Framework

The respective roles of JECFA and the Codex Committee on Food Additives and Contaminants (CCFAC) have been extensively discussed in recent years in the context of the Codex risk analysis framework. In this context, JECFA is regarded as the expert risk assessment body on additives, contaminants and natural toxicants in food, with CCFAC fulfilling the corresponding risk management role. Thus, CCFAC endorses maximum use levels only for those additives for which JECFA (i) has established specifications of identity and purity and (ii) has completed a safety assessment or has performed a quantitative risk assessment

CCFAC also makes recommendations to the Codex Alimentarius Commission on the possible adoption of JECFA specifications as Codex Advisory Specifications. National food control authorities use Codex Advisory Specifications for enforcement purposes, and for ensuring that additives in international commerce meet agreed standards. National governments may also draw on Codex Advisory Specifications when developing their own regulatory standards.

Referrals to JECFA and JECFA/CCFAC Interaction

Additives can be referred to JECFA by CCFAC, Codex member nations, FAO or WHO. However, CCFAC provides, as part of its risk management function, the primary forum for handling these referrals, and the JECFA Secretariat works closely with CCFAC in setting the agendas for JECFA meetings. CCFAC also has an important role in ensuring that those requesting JECFA evaluations understand that they bear responsibility for providing the supporting information, and for submitting this by the date set by JECFA in its pre-meeting Call for Data.

Requests to JECFA may be for a full risk assessment of an additive, in which case the information provided will need to include both toxicology and other safety data, as well as the data required to develop specifications. Alternatively, requests may be limited to a reconsideration of information relating to specifications. In such cases the Committee will, however, bear in mind the need to ensure that the specifications still provide the requisite assurance of safety, and will, therefore, only make revisions that it considers will not affect the validity of the safety evaluation.

The current mechanism for considering JECFA's specifications at CCFAC meetings involves the convening of an *ad hoc* Working Group that meets prior to the plenary CCFAC session. The Working Group, which includes delegates from Codex member states and non-Governmental observers, provides a dedicated forum for the discussion of specifications and other issues relating to the work of JECFA, and the presence of both the JECFA Secretariat and past members of JECFA, attending as part of CCFAC member countries' delegations, provides an opportunity for direct interchange between the risk assessors and risk managers. In this way the Working Group can be seen as fulfilling an important risk communication function in the work of JECFA and CCFAC. The Working Group makes recommendations for consideration at the plenary session of CCFAC, including recommendations on future items to be referred to JECFA and recommendations on which JECFA specifications CCFAC should consider referring to the Codex Alimentarius Commission for possible adoption as Codex Advisory Specifications.

SPECIFICATIONS AND METHODS OF ANALYSIS

Information submitted to JECFA on the identity and purity of food additives should always include details of the relevant analytical methods. Information on the potential compositional variability of the substance should also be given, together with details of any sampling protocols used to assess this. Insufficient information on analytical methodology is one reason why JECFA may be unable to elaborate suitable specifications, or why it may decide that it is only able to assign a 'Tentative' designation, pending receipt of the further information required.

JECFA specifications incorporate guidance on the analytical techniques that should be used to verify the information. Wherever possible, this is done by reference to Volume 4. Otherwise details of the test procedures are set out in the individual specifications monographs.

As JECFA specifications are elaborated for worldwide use, the Committee prefers to quote methods that require the use of apparatus and equipment that is available in most laboratories, provided that such methods are capable of providing results meeting the requirements of the specifications. Methods involving more recently developed techniques or equipment will not normally be quoted until such techniques are accepted internationally and are generally available at reasonable cost. However, reference to specific methods of analysis should not be taken as precluding the use of other methods, provided that these give results of equivalent accuracy and specificity to those quoted.

Changes to analytical methods are reviewed from time to time as part of JECFA's ongoing work. Changes may also be considered when substances are evaluated for the first time, or when new information becomes available on substances that have been previously considered. Changes in analytical methodology may also prompt further consideration of specifications, for example where these changes reveal the possible presence of previously unsuspected contaminants.

FORMAT OF SPECIFICATIONS

Additives other than enzyme preparations and flavouring agents

JECFA specifications for food additives other than enzymes and flavouring agents normally include the headings listed below, which are given in the order in which they appear in the specifications. Note also that aluminium lakes of colouring matters must comply with the *Aluminium Lakes of Colouring Matters - General Specifications* included in Volume 1 of the Compendium.

TITLE

The ***TITLE*** includes the name selected for the individual additive. This is the name that, in the view of JECFA, most appropriately identifies the substance or substances specified. It will normally correspond with the name given to the substance that was subject to safety evaluation, although in some cases there may be discrepancies, as when the substance is a member of a group that has been evaluated collectively, e.g. the phosphates.

In proposing names for additives, those submitting information should consult the guidelines for designating names set out in WHO Technical Report Series 1989, No 776, part 2.3.4. These allow the use of non-proprietary names established by international bodies, or used in national legislation, and indicate that in the absence of these, the name may be chosen from existing common or trivial names of the substance. The selected name must, however, be non-proprietary, and should be distinctive enough to enable the substance to be clearly distinguished from other food additives.

The ***TITLE*** also indicates those cases where the specifications have been designated as 'Tentative'. This designation is used when the Committee regards the information on the additive as insufficient to elaborate fully acceptable specifications, and in such cases the specifications will include an indication of the further information required.

The ***TITLE*** further includes a statement referring to the JECFA meeting at which the specifications were prepared. In cases where the specifications supersede earlier specifications, reference will also be made to these earlier specifications. The statement also includes a reference to the Committee's latest safety evaluation of the substance.

SYNONYMS

Listed in this section are names, acronyms, and abbreviations under which the substance is widely known, other than those used in the ***TITLE*** or in the *Chemical name(s)* (see below). The Codex Alimentarius International Numbering System (INS) number and the USA FD&C number (for colours) are also included here where applicable. Common or trivial names may also be included as synonyms, but registered trade names are not used.

DEFINITION

This section normally includes information on the raw materials used in the manufacture of the additive, together with a brief description of the salient points of the manufacturing method. Proprietary manufacturing information may be

excluded provided that this has been agreed with the JECFA Secretariat. For some substances, for example those of natural origin or those containing a number of different components, more detailed information, including manufacturing and purification methods, is given as necessary. The possible presence of other substances included in commercial additive preparations - for example anti-caking agents and antioxidants – may also be noted.

Chemical name(s)

Where an IUPAC or IUBMB name exists for an additive, this is generally included under this heading and listed first among the chemical names, whether or not it is the systematic name or the recommended common name.

CAS number(s)

The Chemical Abstract Service registry number(s) (CAS number(s)) for the major component(s) of the substance is normally given here. CAS numbers for substances that are encompassed by the specifications, e.g. specific isomeric forms of the main component(s), may also be included along with suitable descriptions.

Chemical formula(e)

The chemical formula(e) of the major component(s) of the additive is given here.

Structural formula(e)

The structural formula(e) corresponding to the major component(s) given under the previous heading is given here, as appropriate.

Formula weight

Formula weights are quoted in JECFA specifications in preference to molecular weights in order to avoid the improper use of the latter form. Formula weights are calculated from values quoted by IUPAC in its Table of Standard Atomic Weights.

Assay

A quantitative assay requirement is provided here, where applicable, to indicate the minimum acceptable content, or maximum acceptable content range, of the principal functional component(s) of the additive.

DESCRIPTION

Information on physical appearance and other significant properties, e.g. stability and odour, is provided in this section, and will normally include any special conditions required for the storage and use of the additive. Such information is by its nature descriptive and should not be interpreted as rigidly as, for example, the requirements under the ***DEFINITION*** section. For safety reasons, descriptions of the taste of a substance are not included.

FUNCTIONAL USES

Functional uses are included in specifications to indicate the technological functions of the additive as used in foods or in food processing. The stated functional uses are not necessarily an exhaustive list, however, and an additive may have uses other than those listed. The functional uses are intended to

conform as far as possible to the harmonized Codex Alimentarius International Numbering System (INS) list of functional uses. Where this is not possible, an unlisted term may be used, and in these cases the new term will be referred for further discussion by the Codex Committee on Food Additives and Contaminants (CCFAC) as part of its consideration of the possible adoption of the specifications as Codex Advisory Specifications.

CHARACTERISTICS

IDENTIFICATION

Identification criteria are generally qualitative and provide part of the means for defining the specified additive. Such criteria typically include solubility in water, solubility in organic solvents, colour reactions, absorption spectra, and pH values.

Where possible, test methods refer to procedures detailed in Volume 4. If the test procedure is not included in the published tests given in Volume 4, it will be included here provided it can be described briefly. Otherwise the details will be set out in the ***TESTS*** section under the heading *IDENTIFICATION TESTS.*

PURITY

Items relating to the purity of the additive, such as limits on impurities and, where appropriate, criteria for microbiological purity, are included under this heading. Limits for trace impurities and for other parameters relating to purity, for example physical properties, are based on the information available on the manufacturing process at the time the specifications were prepared. Limits are set to be consistent with good manufacturing practice and to help provide an assurance of safety, taking into account the use of the additive.

Where possible, test methods refer to procedures detailed in Volume 4. If the test procedure is not included in the published tests given in Volume 4, it will be included here provided it can be described briefly. Otherwise the details of the test will be set out in the ***TESTS*** section under the heading *PURITY TESTS.*

TESTS

IDENTIFICATION TESTS

This section describes in full those test procedures referenced in the ***CHARACTERISTICS*** section under the heading *IDENTIFICATION*. The basic principle behind the analytical method is normally included in the narrative, in addition to details of the apparatus and reagents, the analytical procedure, and the method for calculating results.

PURITY TESTS

This section describes in full those test procedures referenced in the ***CHARACTERISTICS*** section under the heading *PURITY*. The basic principle behind the analytical method is normally included in the narrative, in addition to details of the apparatus and reagents, the analytical procedure, and the method for calculating results. Suppliers of reference standards are named in the text.

METHOD OF ASSAY

The Method of Assay includes a description of the principle of the method, a list of the apparatus and reagents required, details of the analytical procedure, and the

method for calculating results. Where possible, these are described by reference to procedures listed in Volume 4. Suppliers of reference standards are named in the text.

Enzyme preparations

Enzyme preparations used in food processing, whether from animal, vegetable or microbial sources, must comply with the *General Specifications and Considerations for Enzyme Preparations used in Food Processing* found in the next section of this Introduction. In addition, enzyme preparations must meet the specifications criteria contained in the individual specifications monographs These normally include the headings listed below, which are given in the order in which they appear in the specifications.

TITLE

The ***TITLE*** of the specifications monograph includes the name given to the enzyme preparation. This will normally correspond to the name of the enzyme activity or activities that most accurately characterize the preparation. Where appropriate, the source material from which the preparation is derived is also included, so that the name will normally take the form [*Principal enzyme activity/activities]* from *[Systematic IUBMB name of source organism] [Description of genetic modification process where applicable*]. The name may or may not be the same as the systematic name recommended by the IUBMB Enzyme Commission Nomenclature Committee.

The ***TITLE*** also indicates those cases where the specifications have been designated as 'Tentative'. This designation is used when the Committee regards the information on the enzyme preparation as insufficient to elaborate fully acceptable specifications, and in such cases the specifications will include an indication of the further information required and the date by which this must be submitted.

The ***TITLE*** further includes a statement referring to the JECFA meeting at which the specifications were prepared. In cases where the specifications supersede earlier specifications, reference will also be made to these earlier specifications. The statement also includes a reference to the Committee's latest safety evaluation of the substance defined by the specifications.

SYNONYMS

This section includes names and abbreviations under which the preparation is widely known, other than those used in the ***TITLE***. The INS number is also listed where applicable.

SOURCES

This section identifies the animal, plant or microbial sources used to derive the enzyme preparation. The species, strains or variants, strain numbers and plasmid numbers, if from recognized culture collections/depositories (e.g. ATCC), are also given where appropriate. In cases where the source organism has been derived using recombinant DNA technology, a description of this process, including the identity of the host organism, is included.

Active principles

Listed in this section are the principal enzyme activities exhibited by the preparation. IUBMB-recommended names are preferred and will generally be listed first, but other names may also be included.

Systematic names and numbers

Where IUBMB systematic names and Enzyme Commission (EC) enzyme numbers exist, these are listed for each active principle. CAS numbers are also given here where these exist.

Reactions catalysed

This section includes a description of the substrates acted on by the enzyme preparation, the reactions catalysed, and the resultant products.

Secondary enzyme activities

Listed here, if appropriate, are minor enzyme activities that may be present in the enzyme preparation.

DESCRIPTION

Information on physical appearance is provided in this section, together with other information such as solubility in water, solubility in organic solvents, and relevant details of the manufacturing process. Information on the diluents, carriers, stabilisers, preservatives and immobilization agents that may be present in commercial products is also included in this section.

FUNCTIONAL USES

This section gives the principal and secondary technological function(s) of the enzyme preparation as used in foods or in food processing.

GENERAL SPECIFICATIONS

This gives a statement to the effect that all preparations have to conform to the *General Specifications and Considerations for Enzyme Preparations used in Food Processing* (see the next section of this Introduction).

CHARACTERISTICS

IDENTIFICATION

The enzyme activities of the active principles are listed here. Also listed here are criteria for trace impurities resulting from, for example, the use of carriers and immobilization agents, other than those noted in the *General Specifications and Considerations for Enzyme Preparations used in Food Processing* (see the next section of this Introduction).

Where possible, the assay methods refer to procedures detailed in Volume 4. Otherwise the details of the test are set out in the section on ***TESTS***.

TESTS

Assay methods for the enzyme activities of the active principles listed in the ***CHARACTERISTICS*** section under the heading *IDENTIFICATION* are included here in cases where this cannot be done by reference to Volume 4. These describe

the principle of the method, apparatus and reagents required, and give details of the analytical procedure and the method of calculating results. Suppliers of standards for test materials are named in the text.

Flavouring agents

Specifications for flavouring agents are set out in tabular format. They are not included in the Combined Compendium and, as noted above, may be found in the searchable Flavouring Agents database at the FAO website. The specific items included in flavouring agent specifications are set out below.

Specification Heading	Comment
No.	The number allocated by JECFA
Name	The name by which the flavouring agent is known in the trade
Chemical name	The IUPAC name or a similar, more familiar, name
Synonyms	Usually restricted to not more than four names
FEMA	The number allocated by the Flavour and Extract Manufacturers Association of the United States
COE	The number allocated by the Council of Europe. These will be superseded in due course by the European Commission number
CAS	Chemical Abstracts Service registry number
Mol Wt	Molecular weight
Formula	Empirical formula
Physical form/odour	Descriptions of the physical form and odour
Solubility	Solubility in water and in solvents other than ethanol.
Solubility in ethanol	For interpretation see Volume 4
Boiling point (ºC)	At 760 mm Hg, unless specified otherwise
ID Test	Identification test method(s) (IR, NMR, MS)
Assay min	Minimum assay value. Where this is <95%, other components are also specified. See also *Other requirements*
Acid value max	Upper limit for acid value
Refractive index	At 20ºC unless otherwise stated
Specific gravity	At 25ºC unless otherwise stated
Other requirements	May include melting point (mp), content of significant other components, and other significant descriptors
JECFA	Contains a reference to the JECFA meeting at which the substance was considered. The abbreviations used are: N = New specifications; R = Specifications revised; T = 'Tentative' specifications (additional information required); S = Existing specifications maintained; N,T = New, 'tentative' specifications ; S,T = Existing, 'tentative' specifications maintained.
Data required	Listed in the case of 'Tentative' specifications

GENERAL SPECIFICATIONS AND CONSIDERATIONS FOR ENZYME PREPARATIONS USED IN FOOD PROCESSING

The following general specifications were prepared by the Committee at its sixty-seventh meeting (2006) for publication in FAO JECFA Monographs 3 (2006), superseding the general specifications prepared at the fifty-seventh meeting (1) and published in FAO JECFA Monographs 1 (2). These specifications were originally prepared by the Committee at its twenty-fifth meeting (3) and published in FAO Food and Nutrition Papers No. 19 and No. 31/2 (4,5). Subsequent revisions were made by the Committee at its thirty-fifth meeting and published in FAO Food and Nutrition Paper No. 52 (6). Additional amendments were made at the fifty-first meeting and published in FAO Food and Nutrition Paper No. 52 Add. 6 (7), and at the fifty-third meeting (8) and partially published in FAO Food and Nutrition Paper No. 52 Add. 7 (9).

Classification and Nomenclature of Enzymes

Enzymes are proteins that catalyse chemical reactions. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (formerly the International Union of Biochemistry) classified enzymes into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (*10*). Based on the type of reaction catalysed, enzymes are assigned to one of these classes and given an Enzyme Commission (EC) number, a systematic name, and a common name. Other names are also provided, if available. Enzymes used in food processing are often referred to by their common or traditional names such as protease, amylase, malt, or rennet. For enzymes derived from microorganisms, the name of the source microorganism is usually specified, for example, "α-amylase from *Bacillus subtilis*." For enzymes derived from microorganisms modified by using recombinant DNA techniques (referred to as recombinant-DNA microorganisms or genetically modified microorganisms), the names of both the enzyme source (donor organism) and the production microorganism are provided, for example, "α-amylase from *Bacillus licheniformis* expressed in *Bacillus subtilis*."

Enzyme Preparations

Enzymes are used in food processing as enzyme preparations. An enzyme preparation contains an active enzyme (in some instances a blend of two or more enzymes) and intentionally added formulation ingredients such as diluents, stabilizing agents, and preserving agents. The formulation ingredients may include water, salt, sucrose, sorbitol, dextrin, cellulose, or other suitable compounds. Enzyme preparations may also contain constituents of the source organism (i.e. an animal, plant, or microbial material from which an enzyme was isolated) and compounds derived from the manufacturing process, for example, the residues of the fermentation broth. Depending on the application, an enzyme preparation may be formulated as a liquid, semi-liquid or dried product. The colour of an enzyme preparation may vary from colourless to dark brown. Some enzymes are immobilized on solid support materials.

Active Components

Enzyme preparations usually contain one principal enzyme that catalyses one specific reaction during food processing. For example, α-amylase catalyses the hydrolysis of 1,4-α-D-glucosidic linkages in starch and related polysaccharides. However, some enzyme preparations contain a mixture of enzymes that catalyse two or more different reactions in food. Each principal enzyme present in an enzyme preparation is characterized by its systematic name, common name, and EC number. The activity of each enzyme is measured using an appropriate assay and expressed in defined activity units per weight (or volume) of the preparation.

Source Materials

Enzymes used in food processing are derived from animal, plant, and microbial sources. Animal tissues used for the preparation of enzymes should comply with meat inspection requirements and be handled in accordance with good hygienic practice.

Plant material and microorganisms used in the production of enzyme preparations should not leave any residues harmful to health in the processed finished food under normal conditions of use.

Microbial strains used in the production of enzyme preparations may be native strains or mutant strains derived from native strains by the processes of serial culture and selection or mutagenesis and selection or by the application of recombinant DNA technology. Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis (*11–15*). Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Microbial production strains should be taxonomically and genetically characterized and identified by a strain number or other designation. The strain identity may be included in individual specifications, if appropriate. The strains should be maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, should be subjected to methods and culture conditions that are applied consistently and reproducibly from batch to batch. Such conditions should prevent the introduction of microorganisms that could be the source of toxic and other undesirable substances. Culture media used for the growth of microbial sources should consist of components that leave no residues harmful to health in the processed finished food under normal conditions of use.

Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.

Substances used in Processing and Formulation

Substances used in processing and formulation of enzyme preparations should be suitable for their intended uses.

In the case of immobilized enzyme preparations, leakage of active enzymes, support materials, crosslinking agents and/or other substances used in immobilization should be kept within acceptable limits established in the individual specifications.

To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

$$\% \text{ TOS} = 100 - (A + W + D)$$

where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

Purity

Lead: Not more than 5 mg/kg

Determine using an atomic absorption spectroscopy/inductively coupled atomic-emission spectroscopy (AAS/ICP-AES) technique appropriate to the specified level. The selection of the sample size and the method of sample preparation may be based on the principles described in the *Compendium of Food Additive Specifications*, Volume 4.

Microbiological criteria:

Salmonella species: absent in 25 g of sample

Total coliforms: not more than 30 per gram

Escherichia coli: absent in 25 g of sample (Determine using procedures described in the section on Microbiological Analyses, Volume 4)

Antimicrobial activity:

Absent in preparations from microbial sources.

Other Considerations

Safety assessment of food enzyme preparations has been addressed in a number of publications and documents. Pariza & Foster (*11*) proposed a decision tree for determining the safety of microbial enzyme preparations. Pariza & Johnson (*16*) subsequently updated this decision tree and included information on enzyme preparations derived from recombinant-DNA microorganisms. The Scientific Committee on Food (*17*) issued guidelines for the presentation of data on food enzymes. The document includes a discussion on enzymes from genetically modified organisms including microorganisms, plants, and animals. Several international organizations, government agencies, and expert groups have also published discussion papers or guidelines that address safety assessment of food and food ingredients derived from recombinant-DNA plants and microorganisms (*18–28*). Certain information in these documents may be applicable to enzyme preparations derived from recombinant sources.

An overall safety assessment of each enzyme preparation intended for use in food processing should be performed. This assessment should include an evaluation of the safety of the production organism, the enzyme component, side activities, the manufacturing process, and the consideration of dietary exposure. Evaluation of the enzyme component should include considerations of its potential to cause an allergic reaction. For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:

- The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.
- Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance.

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TESTS AND ASSAYS

Descriptions and terms applying to the tests and assays in JECFA specifications include the following:

Analytical Samples

The quantity of the analytical sample to be used is usually indicated in tests and assays. Unless otherwise specified, the quantity used may deviate by 10% from that stated. All quantitative determinations should be conducted on duplicate test portions and in these cases, the amount actually taken should be accurately weighed or measured and the result of the analysis calculated on this exact quantity. When substances are to be "accurately weighed" in a test or assay, the weighing is to be performed in such manner as to limit the error to ±0.1% or less. Quantities smaller than 100 mg should be weighed to the nearest 0.1 mg.

Analytical Standards

Certain procedures (e.g. chromatographic and spectrophotometric instrumental analyses, and antibiotic and enzyme assays) require the use of analytical reference standards. Where suitable standards are available from recognized international bodies, these are specified. In the absence of international standards, it has been necessary in some cases to specify the use of reference standards available from such organizations as the British Pharmacopoeia (BP), Food Chemicals Codex (FCC), National Formulary (NF) of the United States, or the United States Pharmacopeia (USP). The addresses of these organizations may be found in the individual monographs.

Apparatus

With the exception of volumetric flasks and other exact measuring or weighing devices, directions to use a certain size or type of container or other laboratory apparatus are intended only as recommendations, unless otherwise specified. In certain unavoidable cases, the Committee has found it necessary, for accurate description, to use a proprietary name to indicate a certain type of instrument (e.g. spectrophotometer or chromatograph) that is known to give satisfactory results in a particular analytical procedure. However, such a listing in specifications does not necessarily constitute endorsement of the specified instrument by the Committee, nor does it imply that similar instruments from other sources cannot be used with equal or better satisfaction, or that they are of lesser quality or utility than the instrument named.

Blank Tests

The instruction to "perform a blank determination", or similar instructions, indicates that a reagent blank determination should be conducted, in which the same quantities of the same reagents are used, and the same procedure is repeated in every detail, except that the substance being tested is omitted.

Constant Weight

A direction that a substance is to be "dried to constant weight" means that the drying should be continued until two consecutive weighings differ by not more

than 0.5 mg per g of sample taken, the second weighing to follow an additional hour of drying time at the temperature specified. The direction to "ignite to constant weight" means that the ignition should be continued at a temperature of 450°-550°, unless otherwise specified, until two consecutive weighings do not differ by more than 0.5 mg per g of sample taken, the second weighing to follow an additional 30 min ignition period, depending upon the nature of the sample tested.

Desiccants and Desiccators

The expression "in a desiccator" means the use of a tightly closed container of appropriate size and design in which a low moisture content can be maintained by means of a suitable desiccant. Preferred desiccants include, but are not limited to, anhydrous calcium chloride, magnesium perchlorate, phosphorus pentoxide, and silica gel.

Indicators

The quantity of an indicator solution used should be 0.2 ml (approximately 3 drops), unless another quantity is specified.

Odourless

The term "odourless" applies to the examination, after exposure to air for 15 min, of between 1 and 25 g of the substance that has been transferred from the original container to an open evaporating dish of about 100 ml capacity.

Reagents

Reagents used in tests and assays should be of appropriate analytical grade and should contain no interfering impurities.

Significant Figures

Where tolerance limits are expressed numerically, the values are considered to be significant to the number of digits shown. Thus, "not less than 99.0%" means 99.0% but not 99.00%. Values obtained in tests and assays should be rounded off to the nearest indicated digit according to the commonly used practice of rejecting or increasing numbers less than or greater than 5. For example, a requirement of not less than 96.0% would be met by a result of 95.96% but not by a result of 95.94%. When the digit to be dropped is exactly 5, the value should be rounded off to the closest even digit. Thus, both 1.4755 and 1.4765 would be rounded off to 1.476. When a range is stated, the upper and lower limits are inclusive, so that the range consists of the two values themselves, properly rounded off, and all intermediate values between them.

Solutions

All solutions, unless otherwise specified, are to be prepared with distilled or deionized water. Directions for the preparations of "TS" (test solutions), "TSC" (colorimetric solutions) and "PbT" (lead free solutions) are provided in Volume 4. Where volumetric solutions of definite concentration are directed to be used in quantitative determinations, standardized solutions of other concentrations may be employed, unless otherwise specified, if allowance is made for the calculation

factor and if the error of measurement is known not to be increased significantly thereby. Unless otherwise specified, it should be understood that concentrations of solutions prepared from liquids only are expressed in terms of volume in volume (v/v), and solutions of solids in liquids are expressed in terms of weight in volume (w/v). Thus, expressions such as "1 in 10" or "10%" mean that 1 part by volume of a liquid, or 1 part by weight of a solid, is to be dissolved in a volume of the diluent or solvent sufficient to make the finished solutions 10 parts by volume. For other types of solutions (e.g. gases in liquids), and where the above guidelines do not apply, the directions will specify the basis on which the concentration is determined (e.g. w/w, v/w).

Temperatures

Unless otherwise specified, temperatures are expressed in degrees Celsius, and all measurements are to be made at 20°. Ordinary procedures not involving precise instrumental measurements may be conducted at ambient temperature (approximately 15°-30°) unless a particular temperature is specified in a test or assay.

Turbidity

The terms "clear", "almost clear", "very slightly turbid", and "turbid", as specified in Purity Tests for "Clarity and colour of solution", are defined in the individual monographs. The term "no turbidity is produced" means that the clarity of the solution does not change.

Vacuum

The unqualified use of the term "in vacuum" or "in vacuo" means a pressure at least as low as that obtainable by an efficient aspirating water pump (i.e. not higher than about 20 mm of mercury).

Water-bath

The term "water-bath" means a bath of boiling water, unless water at some other temperature is indicated. An alternative form of heating may be employed, provided that the required temperature is approximately maintained and not exceeded.

WEIGHTS AND MEASURES

JECFA generally uses SI units and units accepted for use with SI. The units and abbreviations commonly used in specifications are as follows:

m = metre
cm = centimetre (10^{-2} m)
mm = millimetre (10^{-3} m)
µm = micrometre (10^{-6} m)
nm = nanometre (10^{-9} m)
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)
h = hour(s)*
min = minute(s)*
sec = second(s)
° = degrees Celsius
N = normality (gram equivalents per litre)
M = molarity (moles per litre)
cm^{-1} = wave number
bar = unit of pressure ($kgm^{-1}sec^{-2}$)*
mm Hg = mm of mercury, unit of pressure **
R_f = ratio of (distance of spot from point of application): (distance moved by solvent)
rpm = revolutions per minute

*Time in minutes or hours, and volume in litres or decimals of litres, are outside the SI, but are accepted for use within it. The term 'bar' is also outside the SI, but is subject to review.

** 'mm Hg' is outside the SI system, but is in common use and is retained: 760 mm Hg is equal to 1.013 bar.

ABBREVIATIONS

JECFA specifications include various abbreviations and acronyms, as set out below.

ADI = Acceptable Daily Intake (expressed in mg/kg bw)
AOAC = AOAC INTERNATIONAL, formerly the Association of Official Analytical Chemists
ASTM = ASTM INTERNATIONAL, formerly the American Society for Testing and Materials
ATCC = American Type Culture Collection
CAC = Codex Alimentarius Commission
CAS = Chemical Abstracts System
C.I. = Colour Index
EC = Enzyme Commission of IUBMB (for systematic nomenclature and numbering system of enzymes)
FD&C = Food, Drug and Cosmetic
FCC = Food Chemicals Codex (USA)
FNP = FAO Food and Nutrition Paper
FNS = FAO Food and Nutrition Series
FW = Formula weight
G(L)C = Gas (Liquid) chromatography
(HP)LC = (High Performance) Liquid Chromatography
INS = Codex Alimentarius International Numbering System (for food additives)
ISO = International Organization for Standardization
IUBMB = International Union of Biochemistry and Molecular Biology
IUPAC = International Union of Pure and Applied Chemistry
JECFA = Joint FAO/WHO Expert Committee on Food Additives
i.d. = internal diameter
o.d. = outer diameter
IR = Infrared
meq = milliequivalent
MW = Molecular weight
Mol. Wt = Molecular weight
MTDI = Maximum Tolerable Daily Intake
soln = solution
NMRS = FAO Nutrition Meeting Report Series
TLC = Thin Layer Chromatography
TRS = WHO Technical Report Series
UV = Ultraviolet
VIS = Visible wavelength

VOLUME 4
Combined Compendium of Food Additive Specifications

CONTENTS

GENERAL INFORMATION

Revision of Food and Nutrition Paper No. 5

In 2005, the decision was made by the Food and Agriculture Organization (FAO) to revise and update Food and Nutrition Paper (FNP) No. 5, "Guide to Specifications", containing general notices, general analytical techniques, identification tests, test solutions and other reference materials used in JECFA food additive specifications.

These revisions would be published in Volume 4 of the new Combined Compendium of Food Additive Specifications (FAO JECFA Monographs No. 1).

A number of JECFA and other national experts accepted to draft revisions and updates for selected chapters and other sections of FNP 5. The experts and the chapters or sections that they were requested to revise were as follows:

Dr. Madduri V. Rao (UAE University, United Arab Emirates)
- General Analytical Techniques
- Inorganic Components
- Phosphates
- Sweeteners

Mrs. Inge Meyland (Danish Institute of Food and Veterinary Research, Denmark)
- Appearance and Physical Properties
- Organic Components
- Polyols

Drs. Paul Kuznesof and Julie Barrows (US Food and Drug Administration, USA)
- Food Colours

Dr. Brian Whitehouse (Consultant, United Kingdom)
- Enzyme Preparations
- Flavouring Agents
- Flavour Enhancers

Dr. Richard Cantrill (American Oil Chemists Society, USA)
- Fats and Related Substances
- Hydrocarbons, Waxes and Oils

Dr. Keith Lampel (US Food and Drug Administration, USA)
- Microbiological Methods

In addition, Mr. John Howlett, Consultant, United Kingdom, was asked to prepare proposals for the overall reorganization of the revisions for incorporation into Volume 4.

A Technical Meeting was subsequently held at the Food and Agriculture Organization (FAO) headquarters in Rome, Italy, from 5 to 7 December 2005 to discuss and finalize the proposed revisions and updates prepared by the experts. That meeting was chaired by FAO Consultant John Weatherwax (USA) with FAO Consultant (and JECFA expert) Harriet Wallin (National Food Agency, Finland) as the Rapporteur. All experts involved in preparing revisions participated in the meeting, excepting Dr. Julie Barrows. The FAO JECFA Secretary, Dr. Annika Wennberg also participated.

Criteria for Replacement of Older Methods

The meeting agreed that the following criteria would prompt replacement (if possible) of analytical methods being considered for revision:

- The method includes the use of packed gas chromatography columns. (It was noted that gas chromatographs designed for packed columns are no longer being manufactured).
- The method uses out-dated analytical techniques (e.g. polarography).
- The method includes use of hazardous reagents or solvents (see below).

Older analytical methods that are still in use and are functioning well were not deleted, but references were made to modern methods along with recommendations to use modern techniques.

Those methods which were not referred to by at least one food additive specification were deleted.

Food Chemicals Codex

The Food Chemicals Codex (FCC), published by the National Academies, Washington, D.C., USA, has been an international reference for accepted standards of quality and purity of food chemicals since 1966. The current fifth edition of the FCC has updated many standards and analytical methods, based on changes in science and manufacturing since the previous edition. JECFA has used a number of FCC analytical methods in preparing food additive specifications. FCC methods used in this volume have been taken from the fifth edition.

Hazardous Reagents and Solvents

In some cases, older methods specify the use of solvents or other reagents which are now known to be hazardous to human health. All such materials should be substituted by safer alternatives wherever possible and permitted by the analysis. Where safer substitutes are not available, and no alternative method can be used, the hazardous solvents or reagents should be used with appropriate caution.

Method Validation

All analytical methods referenced in this volume have been shown to provide satisfactory analytical results in normal use. It is incumbent on the analyst, however, to appropriately validate any analytical procedure being used for the first time in their laboratories.

ANALYTICAL TECHNIQUES

CHROMATOGRAPHY

(**Note:** *The following is not intended to represent an exhaustive treatise on chromatographic methods, nor does it take into account numerous variations in procedures which may be necessary, depending upon the nature of analytes, particular reagents or instruments used. Though the fundamental principles on which the instruments work remain the same, the handling and operation of instruments vary from one manufacturer to the other. It is therefore recommended that, for more detailed instructions, the analyst follow the operation and maintenance manuals provided by the instrument manufacturers and directions provided by the supplier of the reagents for the particular additive under analysis.*)

Introduction

Chromatography is defined as an analytical technique whereby a mixture of chemicals may be separated by virtue of their differential affinities for two immiscible phases. One of these, the stationary phase, consists of a fixed bed of small particles with a large surface area, while the other, the mobile phase or "eluant", is a fluid that moves constantly through, or over the surface of, the fixed phase. Chromatographic systems achieve their ability to separate mixtures of chemicals by selectively retaining some compounds in the stationary phase for varying times, while permitting others to move more freely. Therefore, the chromatogram may be evaluated qualitatively by determining the R_f, or retention factor, for each of the eluted substances. The R_f is a measure of that fraction of its total elution time that any compound spends in the mobile phase. Since this fraction is directly related to the fraction of the total amount of the solute that is in the mobile phase, the R_f can be expressed as

$$R_f = V_m C_m/(V_m C_m + V_s C_s),$$

where V_m and V_s are the volumes of the mobile and stationary phase, respectively, and C_m and C_s are the concentrations of the solute in either phase at any time. This can be simplified to

$$R_f = V_m/(V_m + KV_s),$$

where $K = C_s/C_m$ and is an equilibrium constant that indicates this differential affinity of the solute for the two phases. Alternatively, a new constant k', the capacity factor, may be introduced, giving another form of the expression:

$$R_f = 1/(1 + k'),$$

where $k' = KV_s/V_m$. The capacity factor, k', which is normally constant for small samples, is a parameter that expresses the ability of a particular chromatographic system to interact with a solute. The larger the k' value, the more the sample is retained.

Both the retention factor and the capacity factor may be used for qualitative identification of a solute or for developing strategies for improving separation. In terms of parameters easily obtainable from the chromatogram, the R_f is defined as the ratio of the distance travelled by the solute band to the distance travelled by the mobile solvent in a particular time. The capacity factor, k', can be evaluated by the expression

$$k' = (t_r - t_o)/t_o,$$

where t_r, the retention time, is the elapsed time from the start of the chromatogram to the elution maximum of the solute, and t_o is the retention time of a solute that is not retained by the chromatographic system.

Chromatography Principles

Retention of the solutes by the stationary phase may be achieved by one or a combination of mechanisms. Certain substances, such as alumina or silica gel, interact with the solutes primarily by adsorption, either physical adsorption**,** in which the binding forces are weak and easily reversible, or chemisorption, where strong bonding to the surface can occur. Another important mechanism of retention is partition, which occurs when the solute dissolves in the stationary phase, usually a liquid coated as a thin layer on the surface of an inert material or chemically bonded to it.

If the liquid phase is a polar substance (e.g. polyethylene glycol) and the mobile phase is nonpolar, the process is termed normal-phase chromatography. When the stationary phase is nonpolar (e.g. octadecylsilane) and the mobile phase is polar, the process is reversed-phase chromatography. For the separation of mixtures of ionic species, insoluble polymers called ion exchangers are used as the stationary phase. Solute ions in the mobile phase selectively displace an electrically equivalent amount of less strongly bound ions of the ion exchanger in order to maintain the electro neutrality of both phases.

The chromatographic separation of mixtures of large molecules such as proteins may be accomplished by a mechanism called size exclusion chromatography or gel permeation chromatography. The stationary phases used are highly cross-linked polymers that have incorporated a sufficient amount of solvent to form a gel. The separation is based on the physical size of the solutes; those that are too large to fit within the interstices of the gel are eluted rapidly, while the smaller molecules permeate into the pores of the gel and are eluted later.

Separation of certain molecules is accomplished by a mechanism called *affinity chromatography* in which specific binding between an antibody (stationary phase) and antigen (analyte) occur. In any chromatographic separation, more than one of the above mechanisms may be occurring simultaneously.

Chromatography Techniques

Chromatographic separations may also be characterized according to the type of instrumentation or apparatus used. The types of chromatography that may be used are column, paper, thin-layer, gas and high-performance liquid chromatography. Each will be discussed below.

Column Chromatography

Apparatus

The equipment needed for column chromatography is not elaborate, consisting only of cylindrical glass or Teflon tube that has a restricted outflow orifice. The dimensions of the tube are not critical and may vary from 10 to 40 mm in inner diameter and from 100 to 600 mm in length. For a given separation, greater efficiency may be obtained with a long narrow column, but the resultant flow rate will be lower. A fritted-glass disk may be seated in the end

of the tube to act as a support for the packing material. The column is fitted at the end with a stopcock or other flow-restriction device in order to control the rate of delivery of the eluant.

Procedure

The stationary phase is introduced into the column either as a dry powder or as slurry in the mobile phase. Since a homogeneous bed free of void spaces is necessary to achieve maximum separation efficiency, the packing material is introduced in small portions and allowed to settle before further additions are made. Settling may be accomplished by allowing the mobile phase to flow through the bed, by tapping or vibrating the column if a dry powder is used, or by compressing each added portion using a tamping rod. The rod can be a solid glass, plastic, or metal cylinder whose diameter is slightly smaller than the column, or it can be a thinner rod onto the end of which has been attached a disk of suitable diameter. Ion-exchange resins and exclusion polymers are never packed as dry powders since after introduction of the mobile phase they will swell and create sufficient pressure to shatter the column. When the packing has been completed, the sample is introduced onto the top of the column. If the sample is soluble, it is dissolved in a minimum amount of the mobile phase, pipetted onto the column and allowed to percolate into the top of the bed. If it is not soluble or if the volume of solution is too large, it may be mixed with a small amount of the column packing. This material is then transferred to the chromatographic tube to form the top of the bed.

The chromatogram is then developed by adding the mobile phase to the column in small portions and allowing it to percolate through the packed bed either by gravity or under the influence of pressure or vacuum. Development of the chromatogram takes place by selective retardation of the components of the mixture as a result of their interaction with the stationary phase. In column chromatography, the stationary phase may act by adsorption, partition, ion exchange, exclusion of the solutes, or a combination of these effects.

When the development is complete, the components of the sample mixture may be detected and isolated by either of two procedures. The entire column may be extruded carefully from the tube, and if the compounds are coloured or fluorescent under ultraviolet light, the appropriate segments may be cut from the column using a razor blade. If the components are colourless, they may be visualized by painting or spraying a thin longitudinal section of the surface of the chromatogram with colour-developing reagents. The chemical may then be separated from the stationary phase by extraction with a strong solvent such as methanol and subsequently quantitated by suitable methods.

In the second procedure, the mobile phase may be allowed to flow through the column until the components of the mixture successively appear in the effluent. This eluate may be collected in fractions and the mobile phase evaporated if desired. The chemicals present in each fraction may then be determined by suitable analytical techniques.

Paper Chromatography

In this type of chromatography, the stationary phase ordinarily consists of a sheet of paper of suitable texture and thickness. The paper used is made from highly purified cellulose, which has a great affinity for water and other polar solvents since it has many hydroxyl functional groups. The tightly bound water acts as the stationary phase, and therefore the mechanism that predominates is liquid-liquid or partition chromatography. Adsorption of solutes to the cellulose surface may also occur, but this is of lesser importance. Papers especially impregnated to permit ion-exchange or reverse-phase chromatography are also available.

Apparatus

The essential equipment for paper chromatography consists of the following:

- *Vapour-tight chamber*. The chamber is constructed preferably of glass, stainless steel, or porcelain. It is provided with inlets for the addition of solvent or for releasing internal pressure, and it is designed to permit observation of the progress of the chromatographic run without being opened. Tall glass cylinders are convenient if they are made vapour-tight with suitable covers and a sealing compound.
- *Supporting rack*. The rack serves as a support for the solvent troughs and antisiphoning rods. It is constructed of a corrosion-resistant material about 5 cm shorter than the inside height of the chamber.
- *Solvent troughs*. The troughs, made of glass, are designed to be longer than the width of the chromatographic sheets and to contain a volume of solvent greater than that required for one chromatographic run.
- *Antisiphoning rods.* Constructed of heavy glass, the rods are placed on the rack and arranged to run outside of, parallel to, and slightly above the edge of the glass trough.
- *Chromatographic sheets*. Special chromatographic filter paper is cut to length approximately equal to the height of the chamber. The sheet is at least 2.5 cm wide but not wider than the length of the trough. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that when the sheet is suspended from the antisiphoning rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is located a few cm below the rods. Care is necessary to avoid contaminating the paper by excessive handling or by contact with dirty surfaces.

Procedure for Descending Paper Chromatography

Separation of substances by descending chromatography is accomplished by allowing the mobile phase to flow downward on the chromatographic sheet.

The substance or substances to be analyzed are dissolved in a suitable solvent. Convenient volumes of the resulting solution, normally containing 1 to 20 µg of the compound, are placed in 6 to 10 mm spots along the pencil line not less than 3 cm apart. If the total volume to be applied would produce spots of a diameter greater than 6 to 10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added.

The spotted chromatographic sheet is suspended in the chamber by use of the antisiphoning rod and an additional heavy glass rod that holds the upper end of the sheet in the solvent trough. The bottom of the chamber is covered with a mixture containing both phases of the prescribed solvent system. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack or the chamber walls. The chamber is sealed to allow equilibration (saturation) of the chamber atmosphere and the paper with solvent vapour. Any excess pressure is released as necessary. For large chambers equilibration overnight may be necessary.

A volume of the mobile phase in excess of the volume required for complete development of the chromatogram is saturated with the immobile phase. After equilibration of the chamber, the prepared mobile solvent is introduced into the trough through the inlet. The inlet is closed, and the mobile phase is allowed to travel down the paper the desired distance. Precautions must be taken against allowing the solvent to run down the sheet when opening

the chamber and removing the chromatogram. The location of the solvent front is quickly marked, and the sheets are dried.

The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated components of the mixture.

Procedure for Ascending Paper Chromatography

In ascending chromatography, the lower edge of the sheet (or strip) is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.

The test materials are applied to the chromatographic sheet as directed under *Procedure for Descending Paper Chromatography*. Enough of both phases of the solvent mixture to cover the bottom of the chamber are added. Empty solvent troughs are placed on the bottom of the chamber, and the chromatographic sheet is suspended so that the end near which the spots have been added hangs free inside the empty trough.

The chamber is sealed, and equilibration is allowed to proceed as described under *Procedure for Descending Paper Chromatography*. Then the solvent is added through the inlet to the trough in excess of the quantity of solvent required for complete moistening of the chromatographic sheet. The chamber is resealed. When the solvent front has reached the desired height, the chamber is opened and the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.

Small cylinders may be used without troughs so that only the mobile phase is placed on the bottom. The chromatographic sheet is suspended during equilibration with the lower end just above the solvent, and chromatography is started by lowering the sheet so that it touches the solvent.

Detection of Chromatographic Bands

After the chromatogram has been fully developed, the bands corresponding to the various solutes may be detected by means similar to those described in Column Chromatography. If the compounds are coloured or fluorescent under ultraviolet light, they may be visualized directly. Colourless compounds may be detected by spraying the paper with colour-developing reagents. The bands corresponding to the individual components can be cut from the paper, and the chemical substances eluted from the cellulose by the use of a strong solvent such as methanol.

Identification of Solutes

Since the chromatographic mobilities of the solutes may change from run to run due to varying experimental conditions, presumptive identification of a substance should be based on comparison with a reference standard. The R_f values of the unknown substance and the standard on the same chromatogram must be identical. Alternatively, the ratio between the distances travelled by a given compound and a reference substance, the R_r value, must be 1.0. Identification may also be made by mixing a small amount of the reference substance with the unknown and chromatographing. The resulting chromatograph should contain only one spot. Definitive identification of solutes may be achieved by eluting them from the paper and subjecting them to UV, IR, NMR, or mass spectrometry.

Thin-layer Chromatography

In thin-layer chromatography (TLC), the stationary phase is a uniform layer of a finely divided powder that has been coated on the surface of a glass or plastic sheet and that is held

in place by a binder. The capacity of the system is dependent on the thickness of the layer, which may range from 0.1 to 2.0 mm. The thinner layers are used primarily for analytical separations, while the thicker layers, because of their greater sample-handling ability, are useful for preparative work.

Substances that are used as coatings in TLC include silica gel, alumina, cellulose and reversed phase packings. Separations occur due to adsorption of the solutes from the mobile phase onto the surface of the thin layer. However, adsorption of water from the air or solvent components from the mobile phase can give rise to partition or liquid-liquid chromatography. Specially coated plates are available that permit ion-exchange or reverse-phase separations.

Apparatus

Acceptable apparatus and materials for thin-layer chromatography consist of the following:

- *Glass plates:* Flat glass plates of uniform thickness throughout their areas. The most common sizes are 20 x 20 cm and 5 x 20 cm. (Aluminum plates also are commonly used).
- *Aligning tray:* An aligning tray or other suitable flat surface is used to align and hold plates during application of the adsorbent.
- *Adsorbent:* The adsorbent may consist of finely divided adsorbent materials for chromatography. It can be applied directly to the glass plate, or it can be bonded to the plate by means of plaster of Paris or with starch paste. Pretreated chromatographic plates are available commercially.
- *Spreader:* A suitable spreading device that when moved over the glass plate applies a uniform layer of adsorbent of desired thickness over the entire surface of the plate.
- *Storage rack:* A rack of convenient size to hold the prepared plates during drying and transportation.
- *Developing chamber:* A glass chamber that can accommodate one or more plates and can be properly closed and sealed as described under Paper Chromatography. It is fitted with a plate-support rack that can support the plates when the lid of the chamber is in place.
- *UV Chamber:* A UV viewing chamber with eye protection fitted with ultraviolet light source of short (254 nm) and long (360 nm) ultraviolet wavelengths suitable for observations may be required.

Procedure

Clean the plates scrupulously, as by immersion in a chromic acid cleansing mixture, and rinse them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, and then dry.

Arrange the plate or plates on the aligning tray, and secure them so that they will not slip during the application of the adsorbent. Mix an appropriate quantity of adsorbent and liquid, usually water, which when shaken for 30 sec gives a smooth slurry that will spread evenly with the aid of a spreader. Transfer the slurry to the spreader, and apply the coating at once before the binder begins to harden. Move the spreader smoothly over the plates from one end of the tray to the other. Remove the spreader, and wipe away excess slurry. Allow the plates to set for 10 min, and then place them in the storage rack and dry at 105° for 30 min or as directed in the monograph. Store the finished plates in a desiccator.

Equilibrate the atmosphere in the developing chamber as described under the *Procedure for Descending Paper Chromatography* in the section on Paper Chromatography.

Apply the Sample Solution and the Standard Solution at points about 1.5 cm apart and about 2 cm from the lower edge of the plate (the lower edge is the first part over which the spreader moves in the application of the adsorbent layer), and allow to dry. A template will aid in determining the spot points and the 10 to 15 cm distance through which the solvent front should move.

Arrange the plate on the supporting rack (sample spots on the bottom), and introduce the rack into the developing chamber. The solvent in the chamber must be deep enough to reach the lower edge of the adsorbent, but must not touch the spot points. Seal the cover in place, and maintain the system until the solvent ascends to a point 10 to 15 cm above the initial spots, this usually requiring from 15 min to 1 h. Remove the plates, and dry them in air. Measure and record the distance of each spot from the point of origin. If so directed, spray the spots with the reagent specified, observe, and compare the sample with the standard chromatogram.

Detection and Identification

Detection and identification of solute bands is done by methods essentially the same as those described in Paper Chromatography and Column Chromatography. However, in TLC an additional method called fluorescence quenching is also used. In this procedure, inorganic phosphorus is mixed with the adsorbent before it is coated on the plate. When the developed chromatogram is irradiated with ultraviolet light, the surface of the plate fluoresces with a characteristic colour except in those places where ultraviolet-adsorbing solutes are situated. These quench the fluorescence and are detectable as dark spots.

Quantitative Analysis

Two methods are available if quantitation of the solute is necessary. In the first, the bands are detected and their position marked. Those areas of adsorbent containing the compounds of interest are scraped from the surface of the plate into a centrifuge tube. The chemicals are extracted from the adsorbent with the aid of a suitable strong solvent, the suspension is centrifuged, and the supernatant layer is subjected to appropriate methods of quantitative analysis.

The second method involves the use of a scanning densitometer. This is a spectrophotometric device that directs a beam of monochromatic radiation across the surface of the plate. After interaction with the solutes in the adsorbent layer, the radiation is detected as transmitted or reflected light and a recording of light intensity versus distance travelled is produced. The concentration of a particular species is proportional to the area under its peak and can be determined accurately by comparison with standards.

Gas Chromatography

This type of chromatography differs from the others in that the mobile phase is a gas and therefore the solutes must be vaporized in order to allow their movement through the chromatographic column. Gas chromatography is further divided into:

- Gas-solid chromatography, GSC (where stationary phases that are used are solids) or;
- Gas-liquid chromatography, GLC (where stationary phases that are used are liquids).

In gas-solid chromatography, the stationary phase is an active adsorbent, such as alumina, silica, carbon or a polyaromatic porous resin, packed into a column. The passage of solute

through the column will be retarded by adsorption or exclusion mechanisms. In gas-liquid chromatography, the stationary phase is a high boiling point liquid which is finely coated on an inert solid support, such as diatomaceous earth or a porous polymer which is packed into a column (packed column) or is coated as a thin film on the inside of a column (capillary column).

When a volatile compound is introduced into the carrier gas and carried into the column, it is partitioned between the gas and stationary phases by a dynamic countercurrent distribution process. The compound is carried down the column by the carrier gas, retained to a greater or lesser extent by sorption and desorption in the stationary phase. The elution of the compound is characterized by the partition ratio, *k*, a dimensionless quantity also called the capacity factor. It is equivalent to the ratio of the time required for the compound to flow through the column (the retention time) to the retention time of an unretained compound. The value of the capacity factor depends on the chemical nature of the compound; the nature, amount, and surface area of the liquid phase; and the column temperature. Under a specified set of experimental conditions, a characteristic capacity factor exists for every compound. Separation by gas chromatography occurs only if the compounds concerned have different capacity factors

Apparatus

(**Note:** *Most gas chromatographic methods presented in this manual are based on capillary columns as they provide better separation and have replaced the traditional packed columns. As a result, manufacturers of gas chromatographs (GC) have discontinued the manufacture of packed column GCs. However, certain methods using packed columns are listed under individual monographs either due to non-availability of a suitable capillary column or an equivalent method using capillary column has not been developed as yet. In the absence of availability of a packed column GC, analyst may choose an equivalent capillary column, develop and validate proper method for use. The JECFA Secretariat highly appreciates receiving updates on such developments to include in future publications*).

The essential components of a basic gas chromatograph are a carrier gas supply, an injection port, column oven, column, detector, and a suitable data-recording device. The injection port, column and detector are carefully temperature controlled.

Carrier gas supply: Typical carrier gas is helium or nitrogen, depending on the column and detector in use. The gas is supplied from a high-pressure cylinder, suitable pressure or flow controllers are used to regulate the pressure or flow of the carrier gas. Carrier gas shall be highly pure (minimum of 99.999%, water < 1ppm, oxygen <0.1 ppm) and free from any particulate matter. Additionally, gas purifiers such as activated charcoal to remove hydrocarbons, molecular sieve to remove traces of water and oxygen trap may be used to further purify the carrier gas. On line filters (2 μm) may also be used to remove any particulate matter.

Sample injection device: Sample injectors depend on the type of column connected to the injector. They can be classified into packed column injectors (for use with packed columns) and capillary injectors (for use with capillary columns). Sample injection devices range from simple syringes to fully programmable automatic injectors. The amount of sample that can be injected into a capillary column without overloading is small compared with the amount that can be injected into a packed column, and may be less than the smallest amount that can be manipulated satisfactorily by syringe. The injected sample is required to be split into two fractions prior to reaching the column.

Capillary injectors have the capability to split sample into two fractions, a small one that enters the column and a large one that goes to waste (split injector). Injector can be operated either in split mode or splitless mode depending on the quantity of the sample injected. Temperature programmable injectors are also available where the sample is injected into an injector at low temperature (about 50^{o}) and the injector temperature is quickly raised (250^{o}/sec) to the required temperature. This helps in preventing thermal degradation of solutes in the injector.

Compounds to be chromatographed, either in solution or as gases, are injected into the gas stream at the injection port. Depending on the configuration of the instrument, the test mixture may be injected directly into the column or be vaporized in the injection port and mixed into the flowing carrier gas before entering the column. Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are thermally desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample-heating chamber. Solid or liquid samples in tightly closed containers are heated in the chamber for a fixed period of time, allowing the volatile components in the sample to reach equilibrium between the nongaseous phase and the gaseous or headspace phase. After this equilibrium has been established, the injector automatically introduces a fixed amount of the headspace in the sample container into the gas chromatograph.

Column oven: Chromatographic columns are housed in an oven and its temperature is carefully controlled. Column oven may be operated either in isothermal or temperature programming modes. Compounds in the test mixture are separated by virtue of differences in their capacity factors, which in turn depend on their vapor pressure and degree of interaction with the stationary phase. The capacity factor, which governs resolution and retention times of components of the test mixture, is also temperature dependent. The use of temperature programmable column ovens takes advantage of this dependence to achieve efficient separation of compounds differing widely in vapor pressure. As resolved compounds emerge from the column, they pass through a detector, which responds to the amount of each compound present. The type of detector to be used depends on the nature of the compounds to be analyzed.

Columns: Two types of columns, packed or capillary **are** available for use in gas chromatography. Packed columns consist of tubes made up of glass, stainless steel or aluminium which are packed with the stationary phase. Columns of various dimensions may be used, but they usually range from 0.6 to 1.8 m in length and from 2 to 4 mm id.

Capillary columns with 0.25 mm inner diameter and lengths of 30 m or more, have replaced traditional packed columns due to their high efficiency. They are usually made of fused silica or aluminum clad. Fused silica columns are externally coated with polyamide to prevent breakage when they are coiled. Capillary columns are classified into three categories depending on their id. 0.15 – 0.25 id (narrow bore), 0.30-0.53 id (wide bore), .0.53 id (megabore). Wide bore and megabore columns withstand relatively high sample loading as compared to narrow bore columns. The liquid or stationary phase coated is 0.1 to 1.0 mm thick, although nonpolar stationary phases may be up to 5 mm thick. Three types of capillary columns are available:

- Wall coated open tubular columns (WCOT),
- Support coated open tubular columns (SCOT) and:

- Porous layer coated open tubular columns (PLOT).

WCOT columns are the most popular. Because of the absence of a solid support, capillary columns are much more inert than packed columns. Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly proportional to column length, while resolution is proportional to the square root of the column length.

Solid support materials must be as inert as possible in order to prevent interaction of the solutes with active surfaces, resulting in degradation, rearrangement, or loss of peak symmetry (tailing). The most commonly used supports are derived from silicates, usually diatomaceous earth. Before use they are acid-washed, calcined, and treated with a silanizing reagent to render surface hydroxyls inactive. They are available in various particle sizes from 30- to 120-mesh, with the 80- to 100-mesh and 100- to 120-mesh fractions most often used. Porous polymeric materials, which may be coated if desired or used as supplied, are available for the separation of low-molecular-weight compounds.

Liquid phases for partition chromatography may be chosen from a large variety of compounds, ranging from the very polar polyethylene glycols to the nonpolar methyl silicone gums. The choice of a liquid phase for a particular separation is mainly empirical, but usually polar phases are used for the analysis of mixtures of polar compounds. Chemically bonded and cross-lined phases can be used as they withstand higher temperatures (little 'bleeding' at about 300°) and can also be rinsed to restore column performance. They are useful for the analysis of high-boiling-point compounds. Capillary columns with stationary phases with varying polarities, lengths and id are commercially available.

For packed columns, the carrier gas flow rate is usually expressed in milliliters per minute at atmospheric pressure and room temperature. It is measured at the detector outlet with a soap film flow meter while the column is at operating temperature. Unless otherwise specified in the individual monographs, flow rates for packed columns are 60 to 75 ml/min for 4-mm id columns and ~30 ml/min for 2-mm id columns.

Before use, a packed column should be conditioned in the chromatograph to reduce the level of extraneous detector signals produced by the bleeding of volatile substances from the support and the liquid phase. This can be accomplished by increasing the column temperature gradually above its expected operating temperature, while maintaining a low flow of carrier gas through it and leave it overnight at the maximum temperature. During this process, the column should be disconnected from the detector. A suitable test for support inertness should be done.

Capillary columns can be protected by connecting them using a ‘retention gap’ (an empty or low polar capillary column of about 1.5 m in length depending on the polarity of the analytical column) through a quick seal connector. This will retain some unwanted matrix components and protect the column from contamination. For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. Typical linear velocities are 20 to 60 cm/s for helium. At high operating temperatures there is sufficient vapor pressure to result in a gradual loss of liquid phase, a process called ‘‘bleeding.’’

Capillary columns must be tested to ensure that they comply with the manufacturers’ specifications before they are used. These tests consist of the following injections: a dilute methane sample to determine the linear flow velocity; a mixture of alkanes (e.g., C14, C15, and C16) to determine resolution; and a polarity test mixture to check for active sites on the column. The latter mixture may include a methyl ester, an unsaturated compound, a phenol,

an aromatic amine, a diol, a free carboxylic acid, and a polycyclic aromatic compound, depending on the samples to be analyzed.

Detectors: GC detectors can be classified into two groups, universal and selective detectors based on their general response or its response to specific elements or ions. Flame-ionization detector (FID) is the most commonly used detector in GC. The other detectors include electron-capture detector (ECD), thermal conductivity detector (TCD), nitrogen-phosphorus or thermionic specific detector (NPD or TSD), flame photometric detector (FPD) and mass spectrometric detectors (MSD). For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of compound present in the detector over a wide range of concentrations.

- FID has a wide linear range (~10^6) and is sensitive to organic compounds. Unless otherwise specified in individual monographs, FID with either helium or nitrogen carrier gas are to be used for packed columns, and helium is used for capillary columns.
- TCD detects changes in the thermal conductivity of the gas stream as solutes are eluted. Although its linear dynamic range is smaller than that of the FID, it is quite rugged and occasionally used with packed columns, especially for compounds that do not respond to FID.
- NPD contains a thermionic source, such as an alkali-metal salt or a glass element containing rubidium or other metal that results in the efficient ionization of organic nitrogen and phosphorus compounds. It is a selective detector that shows little response to hydrocarbons. This detector can be selectively operated either in the NP mode or P mode by altering the hydrogen flow rate and bead current to achieve better sensitivity of either nitrogen or phosphorus compounds.
- ECD contains a radioactive source (usually 63Ni) of ionizing radiation. It exhibits an extremely high response to compounds containing halogens and nitro groups but little response to hydrocarbons. The sensitivity increases with the number and atomic weight of the halogen atoms.
- FPD can be operated either in phosphorus or sulphur mode by changing the emission filter and selecting proper wavelength for phosphorous or sulphur compounds.

(**Note:** *Refer to the section on gas chromatography- mass spectrometry for details on mass specific detectors*).

Data Collection Devices: Modern data stations receive the detector output, calculate peak areas, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation. Data can also be collected using integrators whose capabilities range from those providing a printout of chromatogram and peak areas/ heights and data stored for possible reprocessing. Simple recorders are available for manual measurement.

Qualitative Analysis

Since it is impracticable in gas chromatography to measure the R_f, presumptive identification of a solute should be done by comparing its position on the chromatogram with that of a reference standard. The position of a solute is characterized by its retention time, the time from injection to the peak maximum; its retention volume, the product of retention time and

carrier gas flow rate; or its retention distance, the distance from injection to the peak maximum. Since conditions may vary between determinations, it is more appropriate to identify a substance by its relative retention,

$$\alpha = (t_2 - t_0)/(t_1 - t_0),$$

Where t_2 is the retention time, volume, or distance of the desired chemical, t_1 is that of the reference compound, preferably determined on the same chromatogram, and t_0 is the retention of an inert compound that is not retained by the column.

A method of definitive identification is to trap and condense the effluent for each peak and subject the condensate to analysis by IR, NMR, mass spectrometry, or other suitable methods.

A measure of the efficiency of a column is the number, N, of theoretical plates it contains for a given compound:

- $N = 16(t_r/w_b)^2$, where t_r is the retention time of the peak, and w_b is its width in units of time at the baseline.

A measure of the efficiency of the separation of two adjacent peaks is given by the dimensionless constant R, the resolution factor, which can be calculated by the equation:

- $R = 2(t_2 - t_1)/(w_1 + w_2)$, where t_2 and t_1 are the retention times of the two peaks, and w_1 and w_2 are the baseline widths determined by the intersection of the tangents of the inflection points of the peaks with the baseline. A resolution of 1.0 corresponds to a peak overlap of approximately 2% and is usually considered to be adequate for analytical purposes.

Quantitative Analysis

In a gas chromatography, the parameter that is proportional to the concentration of any solute is the area under its peak or height. The following techniques can be used for the quantitation of solutes.

Area normalization

This method is based on the assumption that a peak is obtained on the chromatogram for each component of the mixture. The areas of all the peaks, each corrected by multiplying by its response factor, are added together to obtain the total area. Then the percentage of any component is equal to its corrected area divided by the total area and multiplied by 100. This method is reliable only if all components of the sample give a peak and if the various response factors are known.

External standard

A series of standards containing known amounts of the analyte are chromatographed under identical conditions. From the data obtained, a standard or working curve can be constructed by plotting area versus amount of standard that passes through the origin. If the compound to be analyzed is adsorbed within the system, the calibration curve will intersect the abscissa at a nonzero value. This may result in error, particularly for compounds at low concentrations determined by a procedure based on a single reference point. At high concentrations, the liquid phase may be overloaded, leading to loss of peak height and symmetry. A major source of error is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe. Auto samplers provide better precision.

Internal standard

In order to correct for errors that might occur when injection volumes vary or the chromatographic conditions change slightly from run to run, the internal standard method may be used. In this method, another standard, which is chemically similar to the unknown component and which elutes separately from all other peaks, is added in a constant amount to all standard and test solutions of the analyte. After chromatographing, a standard curve is constructed by plotting the area ratios of the standard solutions (area of analyte standard per area of internal standard) versus the weight or concentration ratios of each standard. The unknown is then chromatographed, its area ratio is determined, and the corresponding weight ratio is found by interpolation using the calibration curve. Since the amount of internal standard is constant and known, the concentration of the unknown component can be calculated.

Headspace-Gas Chromatography

Headspace-gas chromatography (headspace-GC) is an analytical technique in which the analyte in its gas phase and its GC analysis have been combined. The method can be applied to the analysis of compounds with a low boiling point, which therefore vapourize easily at low temperatures.

There are two different forms of headspace-GC: static and dynamic headspace-GC. In static headspace-GC the gaseous sample is taken from a sealed headspace vial into a loop and the analytes are transferred to the GC with the help of carrier gas. In dynamic headspace-GC the gaseous sample is forced out of the headspace vial with the help of an external source, usually the same gas as that used as the carrier gas. Headspace injectors are equipped with a thermostatically controlled sample-heating chamber. Solid or liquid samples in tightly closed headspace vials incubated in the chamber for a fixed period of time, allowing the volatile components in the sample to reach equilibrium between the nongaseous phase and the gaseous or headspace phase. After incubation at a specified temperature a gas sample from the head-space vial is sampled.

Purge and Trap Analysis

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are thermally desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

Analysis of Samples - General Method Instructions

(**Note:** *The instructions detailed below provide guidance on performing gas chromatographic analysis. However, analyst should consult individual monographs for sample preparation, appropriate GC column, detector and other conditions*)

The following need careful consideration during the analysis:

Analyte characteristics: The selection of a proper gas chromatographic system depends on the analyte characteristics such as its molecular weight, polarity, boiling point, thermal stability and other properties (refractive index, functional groups, presence of nitrogen, phosphorus, halogens etc. which help in choosing a selective detector).

Column and detector selection: The selection of the right chromatographic column depends on the polarity of analytes and other matrix components. The general rule is that polar compounds can be separated on polar columns. Capillary columns with stationary

phases in different polarities are commercially available. Kovat's index, McReynolds constants and CP Index guide selection of an appropriate column. Choice of selective detectors is based on the analyte characteristics (analytes containing halogens- ECD, nitrogen and phosphorus – NPD/FPD, sulphur-FPD). FID is the most commonly used. The analyst may use either the columns or equivalent as specified in the monograph. Use the detector as specified in the monograph. Use of a different detector requires establishing the limit of detection and limit of determination to ensure that the sensitivity of the method meets the specification requirements.

Optimizing separation: The analyst may follow the GC operating conditions (injector, detector, column temperature - isothermal/temperature programming) detailed in the monographs. Temperature programming is suitable with most capillary columns to achieve optimum separation of analytes and other sample components. However, changes in column length, slight changes in polarity and flow rates demand slight adjustments to the temperatures. It is recommended that the analyst optimize the GC conditions to achieve better separation and peak symmetry by injecting analyte mixture.

Sample preparation: Sample preparation is considered as one of the critical steps in GC analysis and generally involves extraction, cleanup and concentration steps. Sample cleanup involves either single or a combination of steps which include: liquid-liquid extraction, column cleanup, GPC cleanup, SPE cleanup etc. It is recommended to follow the sample preparation method specified in the monograph.

Derivatization: Derivatization is carried out improve volatility, thermal stability, changes in separation properties or paves way for selective detection. General derivatization methods include:

- Methylamine: Trans esterification of carboxylic acids to corresponding methyl esters using sodium methoxide
- Silylation of functional groups [-OH, -COOH, -NH2, =NH, -SH], where the H is replaced by $-O-Si(CH_3)_3$. Common silylation agents are DMCS, TMS, BSTA, BTSFA
- Heptafluoro-derivatives for [-OH] groups - derivatization reagent HFBI.

Most analytes are volatile at the specified GC temperatures and do not require derivatization. It is recommended to follow the derivatization method specified in the monograph.

Qualitative analysis: Comparison of retention times of standard and the analyte is most commonly used for the identification of analytes. Wrong identification is possible in complex matrices where matrix components interfere. Techniques such as use of another column or mass spectral confirmation may be used in such cases. For such cases, specific instructions are detailed in the additive specifications. In the absence of any instructions, follow comparison of retention times for identification.

Quantitative analysis: The external standard method is most commonly used. Instructions are detailed in the additive specifications if alternate methods (normalization technique or internal standard method) are used. Follow the external standard method in the absence of any instructions.

Method validation: It is recommended to validate each test method to ensure accurate and reliable results. Method performance characteristics such as resolution, peak asymmetry, precision, accuracy, limit of detection, limit of determination, linearity and percent recovery provide data on method selectivity, sensitivity and applicability.

Reporting Results: Results need to be reported to three significant figures, unless otherwise specified.

Gas Chromatography – Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry is a hyphenated technique widely used for the confirmation and quantitation of analytes. In this technique, effluents from a GC column are passed into a mass selective detector or mass spectrometer and subjected to analysis. The gas chromatography part is detailed in the above section.

Mass Spectrometry

Mass spectrometry is perhaps the most widely used analytical tool to provide information on molecular weight, identification and confirmation of compounds through their mass spectra as well as quantitation of compounds. In the mass spectrometry, the analyte molecule is bombarded with a stream of electrons that lead to the loss of an electron from the analyte molecule forming a charged molecular ion (M^+). The collision between electrons and analyte molecules usually imparts enough energy to excite the molecules to the higher energy states. Relaxation then often occurs by fragmentation of part of the molecular ion to produce ions of lower masses. The positive ions produced on electron impact are attracted through the slit of a mass spectrometer where they are sorted according to their mass-to-charge ratios and displayed in the form of a mass spectrum.

The plot is in the form of a bar graph that relates the relative intensity (abundance) of mass peaks to their mass-to-charge ratio. The peak having highest intensity is termed as a base peak, and is arbitrarily assigned a value of 100. The heights of the remaining peaks are then computed as a percentage of the base-peak height. Mass spectrometers have the capability to identify base peak and normalize the remaining peaks to that peak. Figure 1 shows a typical mass spectrum of ethyl benzene.

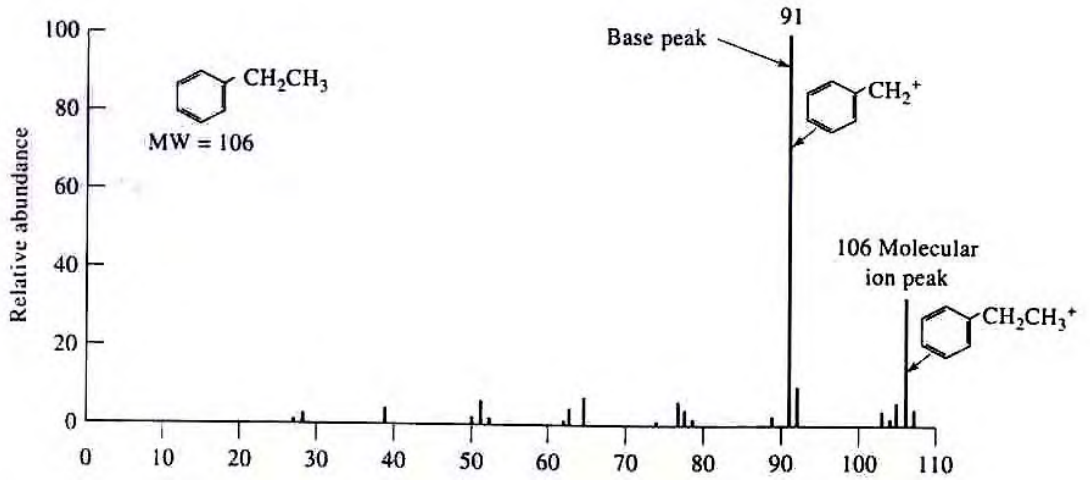


Figure 1

Apparatus:

The essential components of a basic mass spectrometer are a sample inlet system, an ion source, a mass analyzer, a detector, a vacuum system, and a data processing device. Figure 2 shows the block diagram of a typical quadrupole GCMS.

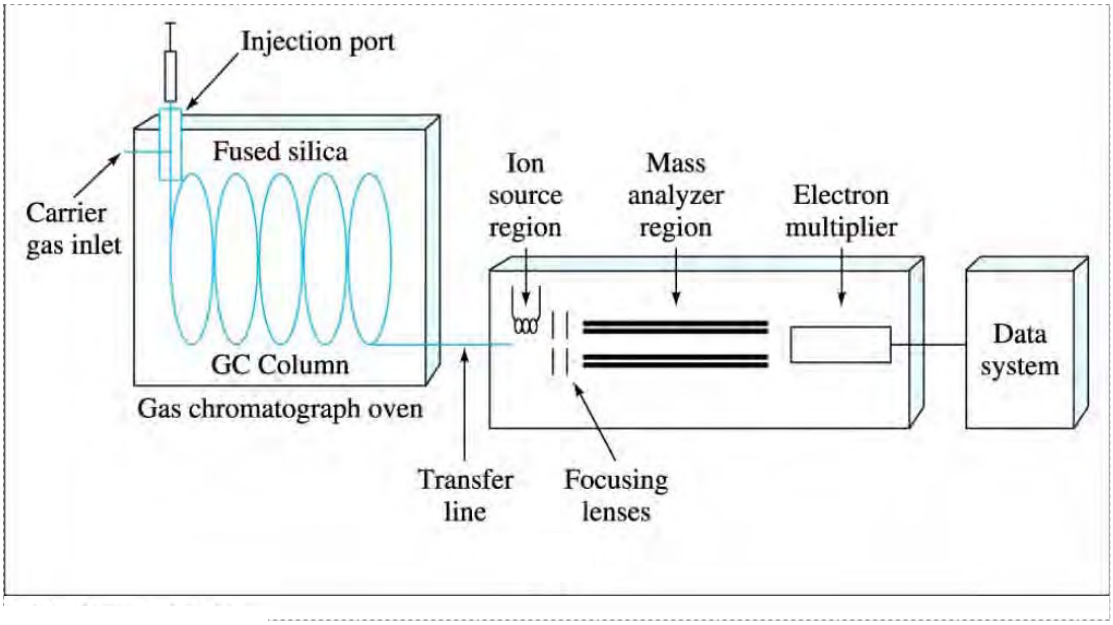


Figure 2

Sample inlet system: The sample inlet system permits introduction of a representative sample into the ion source with minimal loss of vacuum. These include batch inlets, direct probe inlets, chromatographic inlets and capillary electrophoretic inlets. In GCMS, chromatographic inlets are used where the tip of the capillary column is precisely inserted into the inlet.

Ion Source: The purpose of an ion source is to ionize the molecule to produce gaseous analyte ions. Electron impact ionization (EI), chemical ionization (CI) and field ionization (FI) are used to ionize the analytes.

EI is the most common ionization technique used, in which the sample is brought to a temperature high enough to produce molecular vapour, which is then ionized by bombarding with a beam of energetic electrons. Despite certain disadvantages (excessive fragmentation leading to disappearance of molecular ion peak at times and the need to volatilize the sample), this technique produces a reproducible mass spectra of a compound and is the basis on which many mass spectral libraries are built.

In CI, also termed as soft ionization, a gaseous sample is ionized by collision with ions produced by electron bombardment of a reagent gas such as methane or ammonia. Collision between the sample molecule (M) and highly reactive reaction products aroused from the reagent gas usually involves proton or hydride transfer leading to formation of either $(M+1)^+$ or $(M-1)^+$ ions. Relative to EI spectrum, CI spectrum is simple and provides molecular weight information.

Mass Analyzer: The mass analyzer separates the mass fragments produced by the ionization sources. The capability of a mass spectrometer to differentiate between masses is usually stated in terms of its resolution (R) which is defined as $R = m/\Delta m$, where Δm is the mass difference between two adjacent peaks that are just resolved and m is the nominal mass of the first peak (mean of the two peaks is some times used). The mass spectrometer with a resolution of 4000 would resolve peaks with m/z values of 400.0 and 400.1 or 40.00 and 40.01. Several low and high resolution mass analyzers are available which include single stage quadrupole, triple stage quadrupole, ion trap, magnetic sector and time-of-flight.

Single stage quadruple and ion trap are most commonly used. In a quadrupole mass spectrometer, the mass analyzer consists of four parallel cylindrical rods that serve as electrodes, one pair being attached to positive side of a variable dc source and the other pair to the negative terminal. Variable radio-frequency ac potentials are applied to each pair of rods. Meanwhile, ac and dc voltages on the rods are increased simultaneously while maintaining their ratio constant. Fragment ions are accelerated into the space between the rods by a potential of 5 to 10 V. All the ions except those having the m/z value strike the rods and are converted into neutral molecules. Thus, the resonance ions will pass through the quadrupole and reach the transducer. Quadrupole resolves ions that differ in mass by one unit. Quadrupole mass analyzers are termed as mass filters.

A ion trap analyzer consists of a central doughnut-shaped ring electrode and a pair of end cap electrodes. A variable radio-frequency is applied to the ring electrode while the end cap electrodes are grounded. Fragment ions circulate in a stable orbit within the cavity surrounded by the ring. As the radio-frequency energy is increased, the orbits of heavier ions become stabilized, while those with lighter ions become destabilized, causing them to collide with the walls of the ring electrode. When radio-frequency is scanned, the destabilized ions leave the cavity of the ring electrode via openings in the lower end cap and pass into a transducer.

Detectors: Detectors used in the mass spectrometer include electron multiplier detectors, Faraday cup collector and photomultiplier detectors. Electron multiplier detectors are most commonly used.

Vacuum system: The ion source, mass analyzer and the detector must be kept under high vacuum (10^{-4} to 10^{-8} torr) because charged particles interact with components of the atmosphere and are annihilated as a consequence. Several vacuum pumps are used in achieve the required vacuum. Pumps include rotary pumps, oil diffusion pumps and turbo molecular pumps. Differential pumping is recommended using rotary/oil diffusion pump at the first stage and turbo molecular pump at the second stage to crate a proper vacuum environment.

Data processing device: The data processing devices have the capability to control the instrument as well as process a large quantity of data and provide mass spectrum of compounds.

Identification of compounds: Independent identification of compounds is achieved by comparing the sample mass spectrum with several mass spectra available in the mass spectral library. Several EI mass spectral libraries are available. Most modern mass spectrometers have the facility of making custom mass libraries for use. Modern mass spectrometers have the facility to shift from EI to the CI mode which will provide molecular weight information. Tandem mass spectrometry (MS/MS) provides additional confirmation in identification of compounds. In MS/MS, a mass ion (usually the ion with high abundance) is selected and is subjected to further ionization followed by analyzing its mass spectrum. The ion selected for further ionization is termed as parent ion and the ions produced are termed as daughter ions. This process requires a triple quadrupole or ion trap-quadrupole system. In triple quadrupole, the first and third ones work as analyzers, where as the middle one works as an ionizer. The daughter ions so produced are very specific to the parent ions, providing a highly reliable confirmation of the presence of the analyte.

Quantitation: Quantitation is usually carried out using a single ion or a group of ions following selective ion monitoring. The area under the curve for standard as well as sample is used in the quantitation.

High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, exclusion, or ion-exchange processes, depending on the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of nonvolatile organic compounds. Compounds to be analyzed are dissolved in a liquid, and most separations take place at room temperature.

As in gas chromatography, the elution time of a compound can be described by the capacity factor, *k*, which depends on the chemical nature of the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

HPLC is divided into two types namely normal phase chromatography and reversed phase chromatography. In the normal phase chromatography, non-polar to polar stationary phases and non-polar to polar mobile phases separate compounds by their increasing polarity. In the reversed-phase chromatography, the stationary phase has been modified to be a non-polar substance (silica gel has been bonded with a long-chain non polar substance e.g. octadecylsilyl), and polar solvents are used as mobile phase. The order of elution is from polar to non polar compounds.

Reversed-phase chromatography has become a highly powerful technique because its selectivity over a wide range of solutes that can be adjusted by varying the polarity of the mobile phase. In most cases, mobile phases consist of water:methanol or water:acetonitrile. Adjustment of the pH of a mobile phase prevents ionization of weak acids and bases (ion suppression). Addition of an ionic reagent (e.g. heptane-sulfonate) to form a less polar ion pair with a charged solute (ion pairing) aids the retention of polar compounds (e.g. food colours).

Apparatus

The basic components of a liquid chromatograph are a solvent delivery system, a sample injection device, a chromatographic column, a detector, and a suitable data-recording device.

Solvent delivery systems: consists of one or more pumps capable of delivering a pulse-less flow of mobile phase at pressures up to 6000 psig. Flow rates up to 10 ml/min with increments of 0.01 ml/min are typical. In the isocratic mode, where a mobile phase of constant composition is used throughout the run, a single pump and solvent reservoir are required. The mobile phase needs to be prepared externally by mixing the liquids in the required proportion and degassing it by sparging with helium.

For the separation of mixtures where the k' values vary over a wide range, gradient-elution analysis may be used. In this method, the composition of the mobile phase is constantly changed during the chromatographic run. Modern gradient HPLCs (binary, ternary and quaternary systems) have the advantage of internally mixing the liquids, in a mixing chamber, in the required proportions, and have the facility for continuous degassing using a vacuum degasser.

Injectors: After dissolution in a mobile phase or another suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by syringe or loop injectors, or automatically by auto samplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierce able septum and an injection device to transfer sample from the vials to a loop from which it is loaded into the chromatograph. Auto

samplers are programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables. Some valve systems incorporate a calibrated sample loop that is filled with test solution for transfer to the column in the mobile phase. In other systems, a test solution is transferred to a cavity by syringe and then switched into the mobile phase.

Columns used for analytical separations usually have internal diameters of 2 to 4.6 mm and lengths from 5.0 – 30 cm and are usually made of stainless steel. Glass cartridge columns are also used with a cartridge column holder. Larger diameter columns are used for preparative chromatography.

Stationary phases for modern reversed-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3, 5, or 10 μm in diameter. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups. The percent carbon load (amount of bonded phase material loaded on to the silica support, measured as weight percentage of bulk silica) is important in determining the polarity of reversed phase columns. Increasing carbon load and chain length increases retentivity. End capping of columns (a chemical process that reduces the number of free silanol groups attached to the base silica support material) minimizes competing mechanisms.

Columns used for normal phase chromatography are polar in nature (silica). Use of guard columns in front of analytical column protects it and extends its life. Guard columns retain the non-polar substances and any particulate matter that may be in the sample. Guard columns in different sizes (0.5 – 5.0 cm in length) are available for both normal and reversed-phase columns.

Columns are housed in column housing with a thermostatic system to control the temperature. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential stationary phase degradation or mobile phase volatility (Certain resin based columns e.g. carbohydrate columns are heated to 90° to achieve efficient separation of sugars). Unless otherwise specified, columns are used at an ambient temperature.

Ion-exchange chromatography is used to separate water-soluble, ionizable compounds of molecular weights that are less than 2000. The stationary phases are usually synthetic organic resins. Cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines, while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers, all affect the equilibrium. These variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column (total exclusion). Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to remove high molecular weight matrices or to characterize the molecular weight distribution of a polymer or separation of a mixture of proteins with varying molecular weights.

Detectors: The types of detectors most frequently used in HPLC are spectrophotometric, fluorometric, refractometric, potentiometric, voltammetric, or polarographic, electrochemical and mass detectors. The detectors consist of a flow-through cell (8 - 16 μl) to which the column outlet is connected.

In spectrometric detectors, a beam of ultraviolet radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed, variable, and photodiode array (PDA) detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp. Variable wavelength detectors contain a continuous source, such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress.

Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Since the absorption spectrum is continuously collected (from start to the end of the peak) PDA has the additional capability to overlay normalized absorption spectra at different points of the peak and assesses peak purity. Signals also can be extracted at different wavelengths depending on the absorption maximum of each analyte, which provides improved sensitivity. Diode array detectors usually have lower signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate, and temperature, so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done before chromatographic separation or, alternatively, the reagent can be introduced into the mobile phase just before it enters the detector.

Potentiometric, voltammetric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. A pulse-less pump must be used, and care must be taken to ensure that the pH, ionic strength, and temperature of the mobile phase remain constant. Working electrodes are prone to contamination by reaction products with consequent variable responses.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

Mass spectrometric detectors provide additional advantage of independent identification of compounds However, the mobile phase needs to be removed and compounds need to be

ionized. This is achieved by connecting the column outlet to an inlet using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). For further details, refer the section on mass spectrometry.

Data Collection Devices: Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation. Data also may be collected on simple recorders for manual measurement or on stand-alone integrators, which range in complexity from those providing only a printout of peak areas to those providing a printout of peak areas and calculated peak heights plus storing the data for possible use in subsequent reprocessing.

System Suitability

System suitability tests are an integral part of both gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

Figure 3 shows the chromatographic separation of two substances. The resolution, *R*, is a function of column efficiency, *N*, and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the analyte. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components. Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Data from five replicate injections of the analyte are used to calculate the relative standard deviation if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

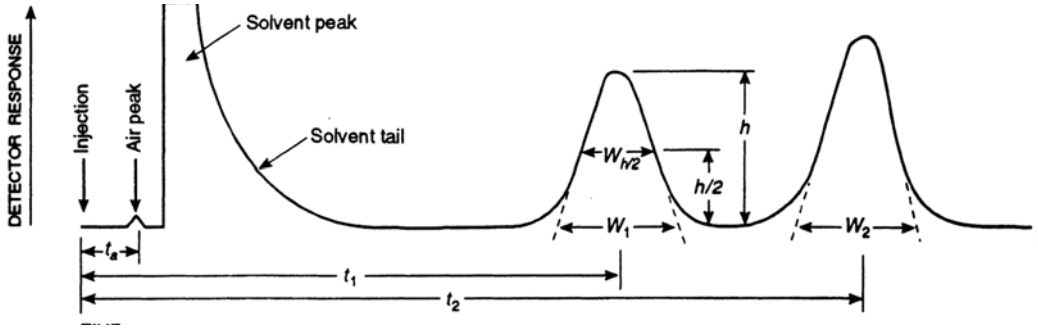


Figure 3: Chromatographic Separation of Two Substances.

Figure 4 shows an asymmetrical chromatographic peak with tailing. The tailing factor, *T*, a measure of peak symmetry, is unity for perfectly symmetrical peaks, and its value increases as tailing becomes more pronounced. In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision, becomes less reliable. The calculation is expressed by the equation

Tailing factor = $T = W_{0.05}/2f$,

Where $W_{0.05}$ is the width of the peak at 0.05 height and f is the width of the first half peak (see details in Figure 4).

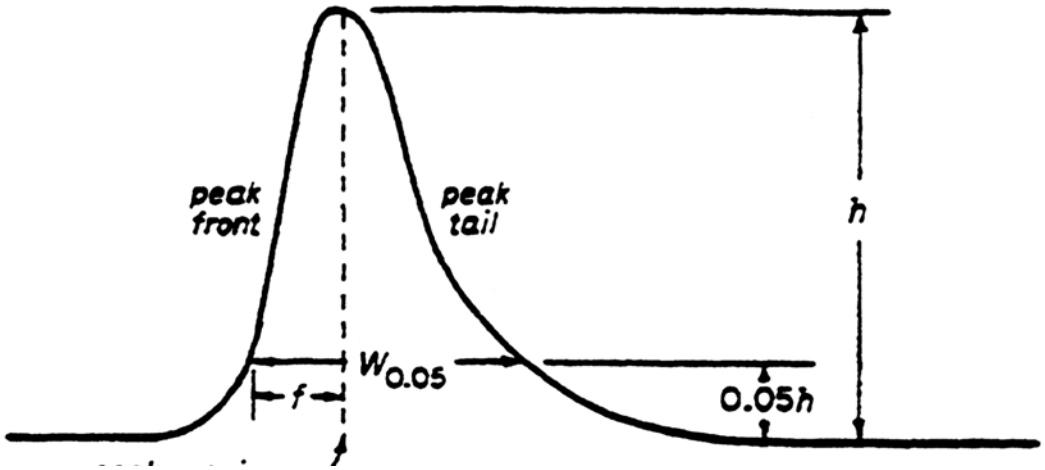


Figure 4: Asymmetrical Chromatographic Peak

These tests are performed by collecting data from replicate injections of standard or other suitable solutions. Adjustments of operating conditions to meet system suitability requirements may be necessary. It is common to determine system suitability parameters from the analyte peak.

To ascertain the effectiveness of the final operating system, it should be subjected to a suitability test before use and during testing whenever there is a significant change in equipment or in a critical reagent or when a malfunction is suspected.

SPECTROPHOTOMETRY AND SPECTROSCOPY

Definitions

Absorption Spectrophotometry

Is the measurement of the selective absorption by molecules or ions, of electromagnetic radiation having a definite and narrow wavelength range, approximating monochromatic light.

Absorption spectrophotometry encompasses the following wavelength and wave number regions: ultraviolet (185 nm to 380 nm), visible (380 nm to 780 nm), near-infrared (0.78 – 2.5 μm or 12,800 - 4,000 cm^{-1}) and mid-infrared (2.5 – 50 μm or 4000 - 200 cm^{-1}).

Colourimetry

Has been commonly accepted as the measurement of "filtered" light in the visible region; however, it is more prudent to restrict its use to those applications where human perception of colour is involved, i.e., the visible region.

Atomic Absorption Spectroscopy

Is the measurement of the radiation absorbed by the unexcited atoms in their gaseous phase, that have been aspirated into a flame or, in the absence of a flame, directly into the path of the radiation.

Flame Emission Spectroscopy (Flame Photometry)

Is the measurement of the intensity of radiation emitted from electronically excited atoms or molecular species. The excitation is brought about by aspirating a solution of the sample into a hot flame.

Fluorescence Spectrophotometry

Or "fluorometry", is the measurement of light emitted from a chemical substance while it is being exposed to electromagnetic radiation. The maximum intensity of the emitted fluorescence is usually at a wavelength longer (i.e., of lower energy) than the exciting radiation.

Turbidimetry and Nephelometry

Are two light-scattering techniques that involve the measurement of light scattered due to its passage through a transparent medium containing a suspended particulate phase. As a result of this scattering, an attenuation or decrease in intensity is suffered by the beam along its axis of travel. Turbidimetry involves the measurement of the degree of attenuation of the light beam by particles suspended in a medium, the measurement being made in the axis of the transmitted beam. Nephelometry involves the measurement of the light scattered by the suspended particles, the measurement being made at right angles to the incident beam.

Terminology

Radiant power, P

Is the energy of radiation per sec that reaches certain areas of a detector. Incident radiant power is usually given the symbol P_o. Alternate terminology is radiation intensity with symbols I and I_o.

Absorbance, $A = Log_{10} (P_o/P)$

Is the logarithm to the base 10 of the quotient of the incident radiant power upon a specimen divided by the radiant power transmitted by the specimen. Former terms were optical density "OD", absorbancy, and extinction.

Specific absorbance, $A^{1\%}_{1\ cm} = A/bc \times 10$

Is the quotient of the absorbance, A, divided by the product of the adsorption path length, b, in cm, and the concentration, c, of the specimen, expressed in g per 100 ml. In general the specific absorbance of a substance is a constant and is independent of the intensity of the incident radiation, path length and concentration. Previously designated by the symbol

$E^{1\%}_{1\ cm}$

Transmittance, $T = (P/P_o)$

Is the quotient of the radiant power transmitted by a specimen divided by the incident power upon the specimen. Transmittance is often expressed as a percentage and is related to the absorbance by the equation $Log_{10}T = A$, or $A = 2 - Log_{10}\%T$. Other terms are transmission and transmittancy.

Absorptivity, $a = A/bc$

Is the quotient of the absorbance, A, divided by the product of the absorption path length, b, in cm, and the concentration, c, of the specimen, expressed in g per 1,000 ml. In general, the absorptivity of a substance is a constant and is independent of the intensity of the incident radiation, path length, and concentration.

Molar absorptivity,

Is the quotient of the absorbance, A, divided by the product of the absorption path length, in cm, and the specimen concentration, expressed in moles per liter. Former terms were molar absorbancy index, molar extinction coefficient and molar absorption coefficient.

Absorption spectrum

Is a graphic representation of the absorbance of a specimen or any of its functions, e.g., transmittance, as the ordinate and the wavelength of the incident radiation as the abscissa.

Fluorescence intensity, I

Is a descriptive term for the fluorescence activity of a substance and is commonly expressed in units related to the detector response. An alternate term is fluorescence power, with the symbol F.

Fluorescence excitation spectrum

Is a graphic representation of the incident (activating) radiation intensity as the ordinate and its wavelength as the abscissa.

Fluorescence emission spectrum

Is a graphic representation of the radiation intensity emitted by an activated species for a specific excitation wavelength as the ordinate and its wavelength as the abscissa.

Turbidance

Is the light-scattering effect of the suspended particles in a turbid medium.

Turbidity

Is a measure of the attenuation in the incident beam power per unit length of a turbid medium. The former term is turbidity coefficient.

Theory and Formulas for Calculations

When electromagnetic radiation travels through a medium containing atoms, molecules, or ions of a chemical substance, radiation at certain frequencies may be partially or totally removed in a process called "absorption". As a result of this absorption, these species are activated from their lowest energy state (ground state) to higher energy states (excited states). For absorption to occur, the energy of the exciting radiation must match the quantized energy difference between the ground state and one of the excited states of the specimen. In atomic absorption, excitation occurs only through electronic transition. Visible and ultraviolet radiation can excite only the outermost or bonding electrons to a higher energy level. Inner-shell electrons are excited only by X-ray radiation (less than 1 nm).

In the case of polyatomic molecules, vibrational and rotational transitions can occur in addition to electronic excitation, and as a result the molecular spectrum consists of closely spaced absorption bands instead of the sharp lines generally observed in the atomic absorption spectrum. Pure vibrational transitions can be achieved by infrared radiation in the range of 1 to 15 μm, while changes in rotational levels are detectable in the region from 10 to 100 μm.

The decrease in the radiant power of a monochromatic beam of light has been found to be proportional to both the distance the radiation travelled through the absorbing medium and the concentration of the absorbing species encountered in that medium. This decrease in energy can be described quantitatively by the Beer-Lambert law:

$$\mathrm{Log}_{10}(P_o/P) = \mathrm{Log}_{10}(1/T) = A = abc$$

Therefore, if the absorptivity and the cell thickness are kept constant during a specific determination, a plot of the absorbance as the ordinate versus concentration as the abscissa should yield a linear relationship. The practical application of the Beer-Lambert law, however, necessitates the use of a reference standard solution of known concentration in order to compare its absorbance with that of the sample solution of unknown concentration. If absorption measurements are conducted in two matching cells having the same path lengths, the absorptivity, a, and the cell thickness, b, will be the same. Therefore the following general formula can be used for the calculation of the unknown concentration of the sample solution,

$$C_u = C_s\,(A_u/A_s)$$

where

C_u = the concentration of the sample solution
C_s = the known concentration of the standard solution
A_u = the absorbance of the sample solution
and A_s = the absorbance of the standard solution.

The Beer-Lambert law is usually satisfactory, provided a thorough understanding of its limitations is taken into consideration. Some of these are of such a fundamental nature that they constitute a real limitation of the law. They are due to the fact that the law does not take into consideration the effects of temperature, wavelength, or solute-solvent and solute-solute interactions, e.g., association, dissociation, chemical reaction, etc. Due to these limitations,

the law usually applies only to dilute solutions, where these interactions are insignificant. Another limitation to the Beer-Lambert law is due to the inability of most instruments to provide monochromatic radiation.

Fluorescence can be observed in a number of gaseous, liquid, or solid substances. However, it is only applied analytically to a relatively small number of organic compounds. Fluorescence occurs when a molecule absorbs sufficient radiation at a certain wavelength to promote it to an excited singlet state with higher levels of energy. The gained energy is released as radiation or "fluorescence" of wavelengths longer than the incident radiation. In most cases, in order for fluorescence to occur the electronic transition involved is a pi → pi* system. To a lesser extent, pi → pi and pi → sigma* transitions occur. There is a delay between the absorption and emission of radiation of about 10^{-9} sec. This short delay period distinguishes fluorescence from phosphorescence, which has a delay period of about 10^{-3} sec and is due to release of weaker radiations from an excited triplet state and not a singlet state as is true of fluorescence. The effect of concentration on the fluorescence intensity can be described by a slightly modified version of the Beer-Lambert law. A linear relationship exists between the fluorescence intensity, I, of the solution and the concentration of the emitting species:

$$I = 2.3K \times bcP_o$$

where K is a constant dependent upon the quantum efficiency of the fluorescence process and instrumental parameters. At constant P_o, a simple relationship as in the Beer-Lambert law can be obtained: I = Kc. Thus a plot of the fluorescence intensity of a solution as the ordinate versus concentration of the emitting species as the abscissa should be linear at low concentrations (see Figure 5).

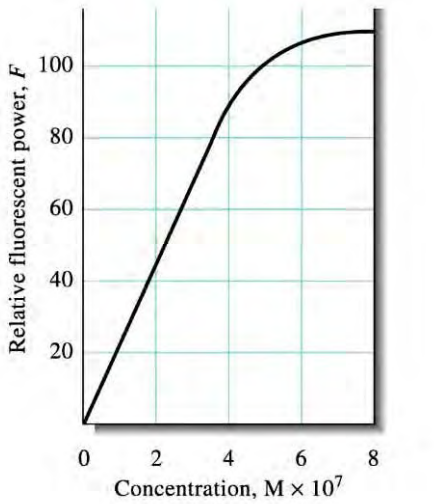


Figure 5

When light passes through a transparent medium containing a suspended particulate phase, scattering occurs in all directions, and as a result the beam loses power along its axis of travel. For dilute suspensions and under fixed conditions (particles, shape, size, refractive index, wavelength of radiation), the loss in radiation intensity can be related to the number of particles (or concentration, c) by an equation similar to the Beer-Lambert law.

$$Log_{10}(P_o/P) = kbc,$$

where tau = kc/2.303. Therefore, in turbidimetric analysis a plot is constructed with standard solutions with $Log_{10}(P_o/P)$ as the ordinate and c as the abscissa (P_o is determined by using the solvent as reference). In nephelometric analysis, the radiation intensity scattered at right angles to the incident beam is plotted as the ordinate versus concentration as the abscissa.

Apparatus

The fundamental principles of optics and electronics that are used in manufacturing spectrophotometers are common to all regions of the spectrum from the vacuum ultraviolet to the far-infrared. However, due to important differences in detail, spectrophotometers are commercially available for use in the visible; in the visible and ultraviolet; in the visible, ultraviolet, and near-infrared; and in the infrared regions of the spectrum. In selecting the type of spectrophotometer to be employed, several factors have to be considered, including the nature of the specimen to be analyzed, the degree of accuracy required, sensitivity, and selectivity.

The essential parts of all spectrophotometers include a stable source of radiant energy; a device that permits the selection of a defined wavelength region such as a prism or grating monochromator: a slit for limiting the suitable bandwidth; a sample compartment ; a radiation detector; and an indicator that may be a meter, a recorder, a digital counter, a printer, or a computer.

Radiation sources commonly employed are hydrogen or deuterium lamps for the ultraviolet region, tungsten lamps for the visible, and a Nernst glower, a globar, or an incandescent wire for the infrared. Quartz or fused-silica cells or cuvettes can be used in the ultraviolet, visible, or near-infrared regions. For infrared spectrophotometry, cells or plates made of sodium chloride are usually used. The radiation detector of ultraviolet and visible radiation is usually a photomultiplier tube with associated amplifiers.

UV/Visible Spectrophotometers:

Two types of spectrophotometers are available: a single-beam spectrophotometer, which adapts well to quantitative analysis that involves single-wavelength measurements, and a double-beam spectrophotometer, which is particularly useful for qualitative analysis and where continuous monitoring of absorbance is required. Some spectrophotometers are manually operated, while others are equipped for automatic and continuous recordings. Spectrophotometers employing the latest technology can be interfaced to a digital computer through an analog-digital converter for the direct determination of different spectra of analytes as well as for the storage of reference spectra. Fourier transformed infrared spectrophotometry is different from the regular dispersion type in that it employs an interferometric technique, whereby polychromatic radiations pass through the specimen to a detector on an intensity and frequency basis. Interfacing with a digital computer is required in order to process such complicated spectral data.

Atomic Absorption Spectrophotometers (AAS):

Instruments for atomic absorption measurements have the same basic components as other spectrophotometers except for the radiation source and the sample container. The most common radiation source is the hollow-cathode lamp, the cathode of which is usually made of the element to be analyzed. The sample is aspirated as a fine mist into a flame that is produced by an optimized mixture of air and acetylene or other suitable gases. The flame vaporizes the solvent molecules and brings the solutes into a gaseous phase. Monochromatic light emitted from the hollow-cathode lamp is selectively absorbed by the analytes.

Photomultiplier tubes are used as detectors, with the electronics designed to accept the modulated radiation source output, thereby negating the continuous signal from the flame. Therefore only changes in the signal from the hollow-cathode lamp are monitored by the detector. These changes are proportional to the number of atoms in the analyte.

Both single-and double-beam atomic absorption spectrophotometers are available. One of the major limitations of Flame AAS is its low sensitivity for certain elements. For determination of elements in low concentrations, electro-thermal atomization technique (graphite furnace atomization) is useful. In the electro-thermal atomization technique, the laminar flow burner is replaced by a graphite furnace. A known volume of analyte solution is placed at the centre of a graphite tube placed in the furnace, the solution is dried and ashed using a controlled heating from electrodes attached to the furnace. Solutes in the dry matter are atomized to a vapour state in a short period to absorb light from the hallow-cathode lamp. Certain elements can be selectively brought to gaseous atomic state (e.g. mercury vapour) without using the flame. Such techniques use vapour generation accessory, in which mercury ions in the solution are reduced to metallic mercury and the mercury vapour is flushed with an inert gas such as nitrogen into an optical cell.

Inductively Coupled Plasma – Atomic Emission Spectrophotometers (ICP-AES):

Instruments for inductively coupled plasma - atomic emission measurements consist of a sample solution aspiration system, a high temperature (~ 6000^{O}) plasma source (a torch in which argon gas is subjected to collisions using a radio frequency source to produce high temperature plasma). The plasma vaporizes the solvent molecule, brings solutes to an atomic state and excites them to higher energy levels. The emitted light from the atoms is measured using a specific emission wavelength. The torch can be operated either in the axial or radial mode depending on the type and nature of elements to be quantitated and type of sample matrix.

Modern ICP instruments use a charge coupled detector (CCD) for the fast measurement of the intensity of emitted light consisting of different wavelengths. Two types of ICP systems are currently available: (a) The sequential system scans the emission spectrum from lower to higher wave lengths during a sample run, making the determination of elements sequentially and; (b) the simultaneous system that collects emission data from all wave lengths simultaneously. ICP instruments have the added advantage of having high linearity of standard curves as compared to AAS and more sensitivity than flame AAS for several elements.

Spectrofluorimeters:

The apparatus for fluorescence intensity measurement is either a fluorometer, which employs filters to restrict the bandwidth of both the excitation and emission beams, or a spectrofluorometer, where prism or grating monochromators are used to limit the excitation beam, the emission beam, or both.

Since a spectrofluorimeter requires a more intense radiation source than a spectrophotometer, either a mercury lamp with its strong discrete lines or a xenon lamp with its energy continuum from the ultraviolet to the infrared is used. Cells for fluorometric measurement are constructed of silica, and the cell compartment is designed to allow a minimum of scattered light to reach the photomultiplier. To minimize scattering interferences, the detector is placed at right angles to the incident excitation beam.

For turbidimetric measurements, a conventional photometer with a tungsten source is usually employed. However, it is preferable to make the measurements in the blue region of a mercury arc. For nephelometric measurements, standard fluorometers are commonly used.

Infrared Spectrometers:

Infrared spectroscopy deals with vibrational and rotational frequencies in the molecules. Infrared measurements may be carried out in the region of near-infrared (0.78 – 2.5 μm or 12,800 - 4,000 cm^{-1}) or mid-infrared (2.5 – 50 μm or 4000 - 200 cm^{-1}). The most commonly used region, however, is 2.5 – 15 μm or 4000 - 670 cm^{-1}.

Two types of instruments are available: (a) dispersive grating spectrophotometers mainly used for qualitative analysis and (b) multiplex instruments, employing Fourier transform that are used for both qualitative and quantitative measurements. The dispersive grating spectrophotometers are replaced by Fourier transform instruments due to their speed, reliability and convenience. Most commercial Fourier transform infrared spectrometers (FTIR) are based on the Michelson interferometer.

The essential components of FTIR include a radiation source, interferometer, sample holder and a detector. Several infrared sources such as Nernst glower, globar source, incandescent wire source, high pressure mercury arc source (used in classical dispersion instruments), and tunable diode laser (helium-neon) sources may be used in FTIR. Michelson interferometer consists of a moving mirror, a fixed mirror and a beam splitter. Radiation from an infrared source is collimated by a mirror and the resultant beam is divided at the beam splitter, half the beam passes to a fixed mirror and the other half is reflected to the moving mirror. After reflection, the two beams recombine at the beam splitter and, for any particular wavelength, constructively or destructively interfere, depending on the difference in optical paths between the two arms of the interferometer. With a constant mirror velocity, the intensity of the emerging radiation at any particular wavelength modulates in a regular sinusoidal manner. In case of a broad band source the emerging beam is a complex mixture of modulation frequencies that after passing through the sample compartment, is focused into the detector. Detectors such as thermal detector (deuterated triglycine sulphate, DTGS) and quantum detector (mercury cadmium telluride, MCT) are used. Detector signal is sampled at precise intervals during the mirror scan. Both the sampling rate and the mirror velocity are controlled by the reference signal incident upon a detector, which is produced by modulation of beam from the helium-neon laser. The resulting signal from the detector is known as a interferogram which contains all the information to reconstruct the spectrum via the mathematical process known as Fourier transformation.

Nuclear Magnetic Resonance Spectrometers (NMR):

Nuclear magnetic resonance spectrometers consist of a continuously spinning super conducting magnet capable of producing a magnetic field (~ 11 tesla) cooled with liquid nitrogen in a double jacketed closed system. Sample solutions using liquid inlets or solids directly using solid inlets are introduced into the core of the magnetic field. The magnetic field produced by the spinning nucleus of elements such as ^{1}H or ^{13}C depend on their environment (functional moieties) in which they exist. Resonance takes place when the magnetic field of the nucleus matches with that of external magnetic field, producing a signal.

The NMR spectrum of a molecule consists of signals produced by a specific nucleus existing in different functional groups (environments) which help in its identification. A study of NMR spectrums helps in the structural elucidation of an unknown molecule. The intensity of the signal produced by a nucleus at a particular resonating frequency is dependent on the

number of such resonating atoms, which makes it possible to quantify the number of atoms and provides a way for the quantitative determination. Instruments ranging from 60 MHz to 1000 MHz are currently available for NMR measurements.

Procedures

Instruction manuals supplied by manufacturers should always be consulted for such matters as care, calibration, handling techniques, and operating procedures. Calibration of both the wavelength and the photometric scales should be conducted at fixed intervals. For wavelength calibration in the ultraviolet and visible regions, a quartz-mercury arc and a holmium oxide glass filter are the most common standards employed. For the near-infrared and infrared regions, a polystyrene film may be used. The photometric scale can be checked by a number of standard inorganic glass filters or by standard solutions of known transmittance.

In absorption spectrophotometry, comparisons of the sample and reference standard are best made at or within ± 1 nm of the wavelength at which maximum absorbance occurs. If matched cells are unavailable, both cells are filled with the selected solvent and any difference in absorbance should be corrected instrumentally or mathematically. The solvent should be transparent in the spectral range of interest. Water, lower alcohols, chloroform, aliphatic hydrocarbons, and many other organic solvents can be used as solvents for ultraviolet and visible measurements. For best results, the concentration of the sample solution should produce an absorbance in the range of about 0.2 to 0.7. For the infrared region, however, few solvents are suitable for sample preparation.

The solvent used in infrared spectrophotometry must not affect the material, usually sodium chloride, of which the cell is made. No solvent in appreciable thickness is completely transparent throughout the infrared spectrum. Infrared spectral grade solvents such as carbon tetrachloride R is practically transparent (up to 1 mm in thickness) from 4,000 to 1,700 cm^{-1} (2.5 to 6 μm). Chloroform R, dichloromethane R, and dibromomethane R are other useful solvents. Carbon disulfide IR (up to 1 mm in thickness) is suitable as a solvent to 250 cm^{-1} (40 μm), except in the 2,400-2,000 cm^{-1} (4.2-5.0 μm) and the 1,800-1,300 cm^{-1} (5.5-7.5 μm) regions, where it has strong absorption. Its weak absorption in the 875-845 cm^{-1} (11,4-11,8 μm) region should also be noted. Other solvents have relatively narrow regions of transparency (carbon disulfide, chloroform, and carbon tetrachloride are the most frequently used). (**Note:** *These solvents are hazardous and appropriate precautions should be taken*).

In some cases, the sample can be dispersed in mineral oil to form a mull, which is transferred to the salt plates. In most cases, however, the sample is dispersed in dried potassium bromide and the mixture is compressed into a tablet or pellet. The proportion of substance to the halide should be about 1 to 200. The amount taken should be such that the weight of substance per area of the disc is about 5-15 μg per mm^2, varying with the molecular weight and to some degree with the type of apparatus used. However, the concentration of the substance should be such that the strongest peak attributable to the substance reaches to between 5% and 25% transmittance. Although the infrared region extends from 2 to 40 μm, for purposes of ascertaining compliance with a reference spectrum, the range from 2.5 to 15 μm (3,800 to 650 cm^{-1}) is usually satisfactory.

For atomic absorption measurements, the solvent should not seriously interfere with the absorption or emission processes or with the production of neutral atoms. Also, both the analyte solution and the standard solution should be as much alike as possible, especially with respect to concentration, viscosity, and surface tension.

In fluorescence spectrophotometry, test solutions are usually very dilute (10^{-3} to 10^{-7}M) in order to minimize the "inner filter" effect caused by significant absorption of incident radiation by the sample near the cell surface. Other undesirable effects of highly concentrated solutions in fluorometry are the "self-quenching" and "self-absorption" phenomena that cause significant deviation from linearity. Test solutions used in fluorometry should also be free from any dust and solid particles, as they cause interference in the measurement. In some cases, before any measurement it is advisable to remove dissolved oxygen from the test solutions, due to its quenching effect. Temperature control is usually needed for extremely sensitive determinations, and baseline correction may be critical.

In turbidimetric and nephelometric measurements, it is important to minimize the settling of the suspended particles. This is generally achieved through the addition of protective colloids.

When visual colour and turbidity comparisons are made, matched colour-comparison tubes that are of the same internal diameter must be used. The solutions to be compared should be at the same temperature (preferably room temperature). For colour comparisons, the tubes are usually held vertically and illuminated from below. Viewing is done from above along the axis of the tube, against a white background. If the colours to be compared are too dark to be viewed downward through the depth of the solutions, they may be viewed horizontally across the diameter of the tubes, with the aid of a light source directed from the back of the tubes. If two layers are present, the designated layer must be viewed horizontally across the diameter of the tube.

For visual turbidity comparisons, the tubes should be viewed horizontally across the diameter of the tubes, with the aid of a light source directed at a right angle against the sides of the tubes.

When conducting limit tests involving the comparison of colour or turbidities, suitable detection instruments may be used in place of the unaided eye.

Applications

Ultraviolet and visible spectra provide only limited information about the chemical structure of a substance. However, because of the sensitivity of these techniques and the high degree of precision and accuracy in their measurements, they are employed extensively in assays and other quantitative determinations.

Near-infrared and infrared spectra, on the other hand, are unique for a given chemical compound, except for optical isomers, which have identical spectra in solution. Polymorphism and other factors, such as variations in crystal size and orientation, the grinding procedure, and the possible formation of hydrates may, however, be responsible for a difference in the infrared spectrum of a given compound in the solid state. The infrared spectrum is usually not greatly affected by the presence of small quantities of impurities (up to several percent) in the tested substance. For identification purposes the spectrum may be compared with that of a reference substance, concomitantly prepared or with a standard reference spectrum. Specificity makes the infrared spectrum one of the most valuable tools for structure elucidation and positive identification of complex organic molecules. Correlation charts and reference spectra of thousands of chemicals are readily available. The sensitivity of infrared analysis, however, is poor (about 1/100 to 1/1,000 of ultraviolet), and therefore it has only a very limited application in quantitative analysis.

Atomic absorption is the technique of choice for the quantitative determination of most of the common elements, even those in complex matrices. Although interferences may occur in the determination of some elements due to chemical interaction between different atoms in the

flame (e.g., cation-anion interference), they can usually be circumvented by preliminary treatment (e.g., addition of a complexing agent) or by the optimization of the instrumentation parameters (e.g., increasing the temperature of the flame to decrease anion-cation attraction). High background signals can be corrected using deuterium background correctors or use of Zeeman furnace techniques. Use of chemical modifiers also helps in reducing background in the furnace analysis.

Fluorescence spectrophotometry has the most inherent sensitivity of all the absorption and light-scattering techniques. Concentrations as low as 10^{-7}M can be quantitatively determined with high precision and accuracy. Fluorescence, however, is not as widespread as the other techniques because of the limited number of organic compounds in which fluorescence can be induced.

Light-scattering techniques, including turbidimetry and nephelometry, are very useful in the determination of weight-average molecular weights in dispersed colloidal systems. Several common ions can be determined using these techniques after their precipitation with suitable reagents. Generally, turbidimetry is adequate for the analysis of heavy suspensions where excessive scattering occurs. Nephelometry, on the other hand, is more suitable for the analysis of cloudy liquids where the attenuation of the radiant power is minimal.

GENERAL METHODS

APPEARANCE AND PHYSICAL PROPERTIES

Boiling Point and Distillation Range

The following method employs 100 ml of sample. In cases where it is necessary or would be desirable to use a smaller sample, the method of McCullough et al. [J. Chem. Ed. 47, 57 (1970)], which employs only 50 µl of sample, may be used.

Definitions

Distillation range: The difference between the temperature observed at the start of a distillation and that observed at which a specified volume has distilled, or at which the dry point is reached.

Initial boiling point: The temperature indicated by the distillation thermometer at the instant the first drop of condensate leaves the end of the condenser tube.

Dry point: The temperature indicated at the instant the last drop of liquid evaporates from the lowest point in the distillation flask, disregarding any liquid on the side of the flask.

Apparatus

Distillation flask: A 200-ml round-bottomed distillation flask of heat-resistant glass is preferred when sufficient sample (in excess of 100 ml) is available for the test. If a sample of less than 100 ml must be used, a smaller flask having the capacity of at least double the volume of the liquid taken may be employed. The 200-ml flask has a total length of 17.9 cm, and the inside diameter of the neck is 2.1 cm. Attached about midway in the neck, approximately 12 cm from the bottom of the flask, is a side arm 12.7 cm long and 5 mm in internal diameter, which is inclined downward at an angle of 75° from the vertical.

Condenser: Use a straight glass condenser of heat-resistant tubing, 56 to 60 cm long and equipped with a water jacket so that about 40 cm of the tubing is in contact with the cooling medium. The lower end of the condenser may be bent to provide a delivery tube or it may be connected to a bent adapter, which serves as the delivery tube.

Note: *All-glass apparatus with standard-taper ground joints may be used alternatively if the assembly employed provides results equal to those obtained with the flask and condenser described above.*

Receiver: The receiver is a 100-ml cylinder, which is graduated in 1-ml sub-divisions and calibrated "to contain". It is used for measuring the sample as well as for receiving the distillate.

Thermometer: A partial-immersion thermometer, calibrated for accuracy, having the smallest practical sub-divisions (not greater than 0.2°) is recommended in order to avoid the necessity for an emergent-stem correction.

Source of heat: A Bunsen burner is the preferred source of heat. An electric heater may be used, however, if it is shown to give results comparable to those obtained with the gas burner.

Shield: The entire burner and flask assembly should be protected from external air currents. Any efficient shield may be employed for this purpose.

Flask support: An asbestos board, 6.5 mm in thickness and having a 10 cm circular hole, is placed on a suitable ring or platform support and fitted loosely inside the shield to ensure that

hot gases from the source of heat do not come in contact with the sides of neck of the flask. A second 6.5 mm asbestos board, at least 225 square cm and provided with a 30 mm circular hole, is placed on top of the first board. This board is used to hold the 200 ml distillation flask, which should be fitted firmly on the board so that direct heat is applied to the flask only through the opening in the board.

Procedure

Note: *This procedure is to be used for liquids that distil above 50° in which case the sample can be measured and received, and the condenser water used, at room temperature (20-30°). For materials boiling below 50°, cool the liquid to below 10° before sampling, receive the distillate in a water bath cooled to below 10° and use water cooled to below 10° in the condenser.*

Measure 100 ± 0.5 ml of the liquid in the 100-ml graduated cylinder and transfer the sample together with an efficient anti-bumping device to the distillation flask. Do not use a funnel in the transfer, or allow any of the sample to enter the side arm of the flask. Place the flask on the asbestos boards, which are supported on a ring or platform, and place in position the shield for the flask and burner. Connect the flask and condenser, place the graduated cylinder under the outlet of the condenser tube and insert the thermometer. The thermometer should be located in the centre of the neck end so that the top of the bulb (when present, auxiliary bulb) is just below the bottom of the outlet to the side arm. Regulate the heating so that the first drop of liquid is collected within 5 to 10 min. Read the thermometer at the instant the first drop of distillate falls from the end of the condenser tube and record as the initial boiling point. Continue the distillation at the rate of 4 or 5 ml of distillate per min, noting the temperature as soon as the last drop of liquid evaporates from the bottom of the flask (dry point) or when the specified percentage has distilled over.

Correct the observed temperature readings for any variation in the barometric pressure from the normal (760 mm Hg) by allowing 0.1° for each 2.7 mm of variation, adding the correction if the pressure is lower, or subtracting if higher than 760 mm Hg. When a total immersion thermometer is used correct for the temperature of the emergent-stem by the formula 0.00015 x N(T - t), in which N represents the number of degrees of emergent-stem from the bottom of the stopper, T the observed temperature of distillation, and t the temperature registered by an auxiliary thermometer the bulb of which is placed midway of the emergent-stem, adding the correction to the observed readings of the main thermometer.

Alternatively, the following simplified correction formula may be applied:

$$t = t_o - k\,(760 - b)$$

in which t_o is the boiling point at 760 mm, b is the observed pressure in mm Hg and k is the correction factor for each 1-mm difference with normal pressure.

The factor k depends on the substance under study; it is given in handbooks and varies between 0.033 and 0.057.

Determination of pH (Potentiometric Method)

The pH of an aqueous solution may be determined accurately by potentiometry using a pH meter. The practical definition of pH in water may be given by the equation:

$$pH = pH_o + [(E - E_o)/0.0591],$$

where pH is the value for the solution being measured, pHo is the value for a standard buffer, E is the potential value for the solution being measured, Eo is the potential value for the

standard buffer, and 0.0591 is the value at 25° of the Nernstian constant. The equation does not apply to solvents other than water, or to mixed solvents that include water. However, the pH meter gives reproducible readings in other solvent systems, on the basis of calibration with aqueous buffers, and while the pH readings lack thermodynamic significance they are useful in setting specifications.

The measurement of pH using a pH meter is a matter of comparing the meter reading of an unknown solution with the meter readings of standard buffers whose pH values are accurately known. Standard buffer solutions are described in compendia, such as the Merck Index. Routine measurement uses only one buffer and an approximation of the electrode slope, usually made by a temperature compensator, pH measurement accurate to ± 0.05 pH unit or better requires the use of two buffers that bracket, if possible, the expected pH range. All samples and buffer should be at the same temperature.

The choice and care of glass and reference electrodes must be carefully considered. The ordinary glass electrode begins to be sensitive to alkali metal cations at pH values above about 9, leading to the so-called alkaline error. Electrodes with a greatly reduced alkaline error should be used for readings in the alkaline range. Store the electrodes in distilled water when not in use, in order to avoid dehydration. "Flow-type" electrodes may be used if evidence of validity of pH measurement with the electrode is demonstrated.

The measurement of the pH of "highly buffered solutions" (distilled water or solutions of non-ionic organic compounds in distilled water) is a particularly difficult measurement. The addition of 0.3 ml of a saturated solution of potassium chloride per 100 ml of distilled water helps by providing a small amount of electrolyte. However, it will usually be necessary to protect the solution being measured from the carbon dioxide in air by use of a blanket of nitrogen during the measurement. Measure the pH of successive portions of the distilled water or test solutions, with vigorous agitation, until the observed results for two successive portions agree within 0.1 pH unit.

Procedure

Use a suitable pH meter and follow the manufacturer's instructions. Each time the electrodes are used, rinse them with distilled or deionised water and carefully blot them dry with clean absorbent tissue. Form a fresh reference electrode liquid junction. Rinse the sample vessel three times with each new solution to be introduced.

Choose two standard buffers (standard buffer solutions are described in compendia, such as the Merck Index) to bracket, if possible, the anticipated pH of the unknown. Warm or cool these standards as necessary to match within 2° the temperature of the unknown, and initially set the temperature compensator to that temperature. Immerse the electrodes in a portion of the first standard buffer, and following the manufacturer's instructions adjust the appropriate standardization control (knob, switch, or button) until the pH reading is that of the buffer. Repeat this procedure with fresh portions of the first standard buffer until two successive readings are within ± 0.02 pH unit without an adjustment of the standardization control.

Rinse the electrodes, blot dry, and immerse them in a portion of the second standard buffer of lower pH. Do not change the setting of the standardization control. Following the manufacturer's instructions, adjust the slope control (thumbwheel switch, knob, or temperature compensator) until the exact buffer pH is displayed.

Repeat the sequence of standardization with both buffers until the pH readings are within ± 0.02 pH unit for both buffers without any adjustment of either control (the amount of sample to be used in sample preparation is given where applicable in the individual specification.). The pH of the unknown solution may then be measured (The difference between the results

of two pH determinations when carried out simultaneously on in rapid succession by the same analyst, under the same conditions, should not exceed 0.05 pH unit.).

Always re-standardize the instrument after even a short period during which the amplifier is turned off.

Melting Range/Melting point

The melting point of a pure substance is the temperature at which the substance changes state from solid to liquid. A substance containing impurities will not melt at one specific temperature, but will melt over a range.

Before determining the melting range of a substance, the sample should be dried under the conditions specified for Loss on Drying in the individual monograph. If a temperature is not specified in the monograph, the sample should be dried for 24 h in a desiccator.

Transfer a quantity of the dried powder to a dry capillary-tube about 10 cm long and sealed at one end (thickness of the wall, 0.10-0.15 mm; i.d. 0.9-1.1 mm) and pack the powder by tapping the tube on a hard surface so as to form a tightly-packed column 2-4 mm in height.

Attach the capillary-tube and its contents to a standard thermometer so that the closed end is at the level of the middle of the bulb, and heat in a suitable apparatus containing an appropriate liquid (liquid paraffin or silicone oil) and fitted with a stirring device and an auxiliary thermometer. Regulate the rise in temperature during the first period to 3° per min. When the temperature has risen to 5° below the lowest figure of the range for the substance being tested, heat more slowly: if no other directions are given, the rate of rise in temperature should be 1-2° per min.

Unless otherwise directed, read the temperature at which the substance is observed to form droplets against the side of the tube and the temperature at which it is completely melted, as indicated by the formation of a definitive meniscus.

To the temperature readings, apply the emergent-stem correction determined as follows:

Before starting the determination of the melting range, adjust the auxiliary thermometer so that the bulb touches the standard thermometer at a point midway between the graduation for the expected melting range and the surface of the heating material. When the substance has melted, read the temperature on the auxiliary thermometer. Calculate the correction to be added to the temperature reading of the standard thermometer from the following formula:

$$0.00015\ N(T - t)$$

in which T is the temperature reading of the standard thermometer, t is the temperature reading of the auxiliary thermometer and N is the number of degrees of the scale of the standard thermometer between the surface of the heating material and the level of the mercury.

The statement "melting range, a^o - b^o" means that the corrected temperature at which the material is observed to form droplets must be at least a^o, and that the material must be completely melted at the corrected temperature b^o.

Refractive Index

The refractive index of a transparent substance is the ratio of the velocity of light in air to its velocity in that material under like conditions. It is equal to the ratio of the sine of the angle of incidence made by a ray in air to the sine of the angle of refraction made by the ray in the

material being tested. The refractive index values specified are for the D line of sodium (589 nm) unless otherwise specified.

The determination should be made at the temperature specified in the individual monograph or at 25° if no temperature is specified. This physical constant is used as a means for identification of, and detection of impurities in, volatile oils and other liquid substances. The Abbé refractometer, or other refractometers of equal or greater accuracy, may be employed at the discretion of the operator.

Solidification Point

This method is designed to determine the solidification point of food grade chemicals having appreciable heats of fusion. It is applicable to chemicals having solidification points between -20° and +150°.

Solidification point is an empirical constant defined as the temperature at which the liquid phase of a substance is in approximate equilibrium with a relatively small portion of the solid phase. It is measured by noting the maximum temperature reached during a controlled cooling cycle after the appearance of a solid phase.

Solidification point is distinguished from freezing point in that the latter term applies to the temperature of equilibrium between the solid and liquid state of pure compounds. Some chemical compounds have two temperatures at which there may be a temperature equilibrium between solid and liquid state depending upon the crystal form of the solid that is present.

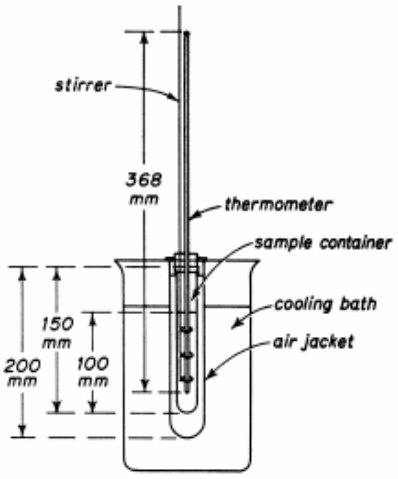
Apparatus

The apparatus is illustrated below and consists of the components described as follows:

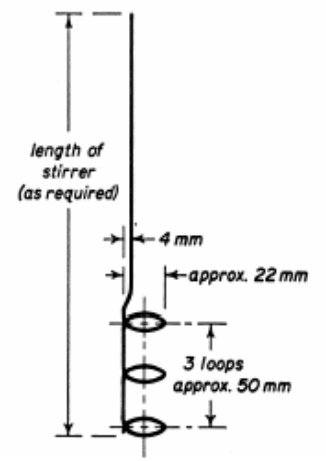
Sample container: Use a standard glass 25 x 150 mm test-tube with lip, fitted with a stopper bored to hold the thermometer in place and to allow stirring with stirrer.

Thermometer: A thermometer having a range not exceeding 30° graduated in 0.1° divisions, and calibrated for accuracy at 76 mm immersion, should be employed. A thermometer should be so chosen that the stopper of the sample container does not obscure the solidification point.

Stirrer: The stirrer consists of a 1 mm diameter (B & S gauge 18) corrosion-resistant wire bent in a series of 3 loops about 25 mm apart. It should be made so that it will move freely in the space between the thermometer and the inner wall of the sample container. The shaft of the stirrer should be of a convenient length designed to pass loosely through a hole in the stopper holding the thermometer. Stirring may be hand-operated or mechanically activated at 20 to 30 strokes per min.



Apparatus
for determining Solidification Point



Stirrer
for Solidification Point determination

Air jacket: Use a standard glass 38 x 200 mm test-tube with lip, fitted with a stopper bored with a hole into which the sample container can easily be inserted up to the lip.

Cooling bath: Use a 2-L beaker or similar suitable container as a cooling bath. Fill it with an appropriate cooling medium such as glycerine, mineral oil, water, water and ice or alcohol-dry ice.

Assembly: Assemble the apparatus in such a way that the cooling bath can be heated or cooled to control the desired temperature ranges. Clamp the air jacket so that it is held rigidly just below the lip and immerse it in the cooling bath to a depth of 160 mm.

Preparation of sample: The solidification point is usually determined on chemicals as they are received. Some may be hygroscopic, however, and require special drying. Where this is necessary it will be noted in the monograph. Products which are normally solid at room temperature must be carefully melted at a temperature about 10° above the expected solidification point. Care should be observed to avoid heating in such a way as to decompose or distil any portion of the sample.

Procedure

Adjust the temperature of the cooling bath to about 5° below the expected solidification point. Fit the thermometer and stirrer with a stopper so that the thermometer is centred and the bulb is about 20 mm from the bottom of the sample container. Transfer a sufficient amount of the sample, previously melted if necessary, into the sample container to fill it to a depth of about 90 mm when in molten state. Place the thermometer and stirrer in the sample container and adjust the thermometer so that the immersion line will be at the surface of the liquid and the end of the bulb 20 ± 4 mm from the bottom of the sample container. When the temperature of the sample is about 5° above the expected solidification point, place the assembled sample tube in the air jacket.

Allow the sample to cool while stirring at the rate of 20 to 30 strokes per min, in such a manner that the stirrer does not touch the thermometer. Stir the sample continuously during the remainder of the test.

The temperature at first will gradually fall, then become constant as crystallization starts and continues under equilibrium conditions, and finally will start to drop again. Some chemicals may super-cool slightly below (0.5°) the solidification point; as crystallization begins the temperature will rise and remain constant as equilibrium conditions are established. Other products may cool more than 0.5° and cause deviation from the normal pattern of temperature changes. If the temperature rise exceeds 0.5° after the initial crystallization begins, repeat the test and seed the melted compound with small crystals of the sample at 0.5° intervals as the temperature approaches the expected solidification point. Crystals for seeding may be obtained by freezing a small sample in a test-tube directly in the cooling bath. It is preferable that seeds of the stable phase be used from a previous determination.

Observe and record the temperature readings at regular intervals until the temperature rises from a minimum, due to super-cooling, to a maximum and then finally drops. The maximum temperature reading is the solidification point. Readings 10 sec apart should be taken in order to establish that the temperature is at the maximum level and continues until the drop in temperature is established.

Solubility

Approximate solubilities, as specified in the Identification Tests, are to be interpreted according to the following descriptive terms:

Descriptive term	Parts of solvent required for 1 part of solute			
Very soluble	Less than 1			
Freely soluble	From	1	to	Less than 10
Soluble	From	10	to	Less than 30
Sparingly soluble	From	30	to	Less than 100
Slightly soluble	From	100	to	Less than 1,000
Very slightly soluble	From	1,000	to	Less than 10,000
Practically insoluble or in soluble	More than 10,000			

Procedure: Unless otherwise specified, transfer a known amount of the sample into a flask containing known amount of the specified solvent, shake for no less than 30 sec and no more than 5 min.

Specific Gravity

Specific gravity is defined as the ratio of the mass of the sample to the mass of an equal volume of the standard material. The specific gravity (d^t_t) means the ratio of the weight of the sample at t'° to that of an equal volume of water at t°. Unless otherwise specified, specific gravity is noted as D^{20}_{20}. Specific gravity is determined by one of the following methods, unless otherwise specified.

Measurement by Pycnometer

A pycnometer is a vessel made of glass with a capacity of usually 10 to 100 ml. It has a ground-glass stopper fitted with a thermometer, and has a side tube with a mark and a ground-glass cap. Weigh a pycnometer previously cleaned and dried, and note the weight W. Remove the stopper and the cap, fill the pycnometer with a sample, keep at the temperature of about 1° to 3° lower than that specified, and stopper, taking care not to leave bubbles.

Raise the temperature gradually until the thermometer shows the specified temperature. Remove the sample above the mark from the side tube, replace the cap, and wipe the outside thoroughly. Weigh, and note the weight W1. Using the same pycnometer, perform the similar determination with water. Weigh the pycnometer containing water at the specified temperature, and note the weight W2. Calculate the specific gravity of the sample by the following formula.

$$d = (W1 - W) / (W2 - W)$$

Measurement by Mohr-Westphal Balance

Keep the balance horizontal; attach the glass tube in which a thermometer is enclosed by a wire onto the end of the arm. Immerse the glass tube in water in a cylinder, place the largest rider on the arm at the mark 10, and adjust the balance by moving the nut at the specified temperature.

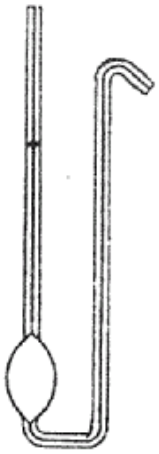
After that, immerse the glass tube in the sample, adjust the balance by hanging riders on the arm, and read the specific gravity at the marks at which riders are placed. It is necessary to make the length of the part of wire that is immersed in a sample equal to that immersed in water by changing the height of the sample in the cylinder.

Measurement by Hydrometer

Use a hydrometer with a precision intended for use at the specified temperature. Clean the hydrometer with alcohol. Shake the sample well, and place in the hydrometer after bubbles have disappeared. At the specified temperature, when the hydrometer has settled, read the specific gravity at the upper rim of the meniscus. In case of any hydrometer, however, for which special directions are given, follow the directions.

Measurement by Sprengel-Ostwald Pycnometer

A Sprengel-Ostwald pycnometer (see figure) is a vessel made of glass with a capacity of usually 1 to 10 ml. As shown in the figure, both the ends are thick-walled fine tubes one of which has a mark on it. A platinum or an aluminium wire is attached to hang on the arm of a chemical balance.



Sprengel-Ostwald Pycnometer

Weigh the pycnometer, previously cleaned and dried (W). Immerse the curved tube in the sample kept at a temperature 3° to 5° lower than the specified temperature, attach a rubber

tube at the end of the straight tube, and suck the sample gently until it comes up above the mark, taking care to prevent formation of bubbles. Immerse the pycnometer in a water bath kept at the specified temperature for about 15 min, and by attaching a piece of filter paper at the end of the curved tube, adjust the end of the sample to the mark. Remove the pycnometer from the water bath, and wipe the outside well. Weigh and note the weight W1. By using the same pycnometer, perform the same determination with water. Weigh the pycnometer containing water at the specified temperature, and note the weight W2. Calculate the specific gravity by the following formula:

$$d = (W1 - W) / (W2 - W)$$

Specific Rotation

Optical rotation of chemicals is generally expressed in degrees, as either "angular rotation" (observed) or "specific rotation" (calculated with reference to the specific concentration of 1 g of solute in 1 ml of solution, measured under stated conditions).

Specific rotation usually is expressed by the term $[\alpha]t_x$, in which t represents, in degrees centigrade, the temperature at which the rotation is determined, and x represents the characteristic spectral line or wavelength of the light used. Spectral lines most frequently employed are the D line of sodium (doublet at 589.0 and 589.6 nm and the yellow-green line of mercury at 546.1 nm). The specific gravity and the rotatory power vary appreciably with the temperature.

The accuracy and precision of optical rotation measurements will be increased if they are carried out with due regard for the following general considerations.

The source of illumination should be supplemented by a filtering system capable of transmitting light of a sufficiently monochromatic nature. Precision polarimeters generally are designed to accommodate interchangeable disks to isolate the D line from sodium light or the 546.1 nm line from the mercury spectrum. With polarimeters not thus designed, cells containing suitably coloured liquids may be employed as filters [see "Technique of Organic Chemistry", A. Weissberger. Vol. I, Part II, 3rd ed. (1960), Interscience Publishers, Inc., New York, N.Y.].

Special attention should be paid to temperature control of the solution and of the polarimeter. Observations should be accurate and reproducible to the extent that differences between replicates, or between observed and true values of rotation (the latter value having been established by calibration of the polarimeter scale with suitable standards), calculated in terms of either specific rotation or angular rotation, whichever is appropriate, shall not exceed one-fourth of the range given in the individual monograph for the rotation of the article being tested. Generally, a polarimeter accurate to 0.05° of angular rotation, and capable of being read with the same precision, suffices. In some cases, a polarimeter accurate to 0.01° or less, of angular rotation, and read with comparable precision, may be required.

Polarimeter tubes should be filled in such a way as to avoid creating or leaving air bubbles, which interfere with the passage of the beam of light. Interference from bubbles is minimized with tubes in which the bore is expanded at one end. However, with tubes of uniform bore, such as semimicro-or micro-tubes, care is required for proper filling. At the time of filling, the tubes and the liquid or solution should be at a temperature not higher than that specified for the determination, to guard against the formation of a bubble upon cooling and contraction of the contents.

In closing tubes having removable end-plates fitted with gaskets and caps, the latter should be tightened only enough to ensure a leak-proof seal between the end-plate and the body of the

tube. Excessive pressure on the end-plate may set up strains that result in interference with the measurements. In determining the specific rotation of a substance of low rotatory power, it is desirable to loosen the caps and tighten them again between successive readings in the measurement of both the rotation and the zero-point. Differences arising from end-plate strain thus generally will be revealed and appropriate adjustments to eliminate the cause may be made.

Procedure

In the case of a solid, dissolve the substance in a suitable solvent, reserving a separate portion of the latter for a blank determination. Make at least five readings of the rotation of the solution, or of the substance itself if liquid, at 25° or the temperature specified in the individual monograph. Replace the solution with the reserved portion of the solvent (or, in the case of a liquid, use the empty tube), make the same number of readings, and use the average as the blank value. Subtract the blank value from the average observed rotation if the two figures are of the same sign, or add if opposite in sign, to obtain the corrected angular rotation.

Calculation

Calculate the specific rotation of a liquid substance, or of a solid in solution, by application of one of the following formulas:

- For liquid substances, $[\alpha]_x^t$ = (a/ld);
- For solutions of solids, $[\alpha]_x^t$ = (100a/lpd) = (100a/lc);

in which a is the corrected angular rotation, in degrees, at temperature t; l is the length of the polarimeter tube in decimeters; d is the specific gravity of the liquid or solution at the temperature of observation; p is the concentration of the solution expressed as the number of g of substance in 100 g of solution; and c is the concentration of the solution expressed as the number of g of substance in 100 ml of solution.

The concentrations p and c should be calculated on the dried or anhydrous basis, unless otherwise specified.

IDENTIFICATION TESTS

The following are chemical tests to identify specific inorganic ions or organic moieties. Test solutions (TS) used are defined under the section on Media, Reagents and Solutions.

Inorganic Ions

Aluminium

Solutions of aluminum salts yield with ammonia TS a white, gelatinous precipitate which is insoluble in an excess of ammonia TS. A similar precipitate is produced by sodium hydroxide TS or sodium sulfide TS, but it dissolves in an excess of either reagent.

Ammonium

Sodium hydroxide TS decomposes ammonium salts with the evolution of ammonia, recognizable by its odour and its alkaline effect upon moistened red litmus paper. The decomposition is accelerated by warming.

Bicarbonate

See Carbonate.

Bisulfite

See Sulfite.

Bromate

Solutions of bromates acidified with nitric acid (1 in 20), yield a white, crystalline precipitate with the addition of 2 or 3 drops of silver nitrate TS, which dissolves by heating. A pale yellow precipitate is produced with the addition of 1 drop of sodium nitrite TS.

Solutions of bromates acidified with nitric acid (1 in 20), produce a yellow to reddish brown colour with the addition of 5 or 6 drops of sodium nitrite TS. With the addition of 1 ml of chloroform and stirring, the chloroform layer becomes a yellow to reddish brown colour.

Bromide

Free bromine is liberated from solutions of bromides upon the addition of chlorine TS, dropwise. When shaken with chloroform, the bromine dissolves, colouring the chloroform red to reddish brown. A yellowish white precipitate, which is insoluble in nitric acid and slightly soluble in ammonia TS, is produced when solutions of bromides are treated with silver nitrate TS.

Calcium

Insoluble oxalate salts are formed when solutions of calcium salts are treated in the following manner: using 2 drops of methyl red TS as indicator, neutralize a solution of a calcium salt (1 in 20) with ammonia TS. A white precipitate of calcium oxalate forms upon the addition of ammonium oxalate TS. This precipitate is insoluble in acetic acid but dissolves in hydrochloric acid.

Calcium salts moistened with hydrochloric acid impart a transient yellowish red colour to a non-luminous flame.

Carbonate

Carbonates and bicarbonates effervesce with acids, yielding a colourless gas (carbon dioxide) which produces a white precipitate immediately when passed into calcium hydroxide TS. Cold solutions of soluble carbonates are coloured red by phenolphthalein TS, whereas solutions of bicarbonates remain unchanged or are slightly changed.

Chloride

Solutions of chlorides yield with silver nitrate TS a white, curdy precipitate which is insoluble in nitric acid but soluble in a slight excess of ammonia TS. Chlorine, recognizable by its distinctive odour, is evolved when solutions of chloride are warmed with potassium permanganate and dilute sulfuric acid TS.

Copper

When solutions of cupric compounds are acidified with hydrochloric acid, a red film of metallic copper is deposited upon a bright untarnished surface of metallic iron. An excess of ammonia TS, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-coloured solution. Solutions of cupric salts yield with potassium ferrocyanide TS a reddish brown precipitate, insoluble in dilute acids.

Ferric salts

Potassium ferrocyanide TS produces a dark blue precipitate in acid solutions of ferric salts. With an excess of sodium hydroxide TS, a reddish brown precipitate is formed. Solutions of ferric salts produce with ammonium thiocyanate TS a deep red colour which is not destroyed by dilute mineral acids.

Ferrocyanide

To 10 ml of a 1% solution of the sample add 1 ml of ferric chloride TS. A dark blue precipitate is formed.

Ferrous salts

Potassium ferricyanide TS produces a dark blue precipitate in solutions of ferrous salts. This precipitate, which is insoluble in dilute hydrochloric acid, is decomposed by sodium hydroxide TS. Solutions of ferrous salts yield with sodium hydroxide TS a greenish white precipitate, the colour rapidly changing to green and then to brown when shaken.

Iodide

Solutions of iodides, upon the addition of chlorine TS, dropwise, liberate iodine which colours the solution yellow to red. Chloroform is coloured violet when shaken with this solution. The iodine thus liberated gives a blue colour with starch TS. Silver nitrate TS produces in solutions of iodides a yellow, curdy precipitate which is insoluble in nitric acid and in ammonia TS.

Iron

Solutions of ferrous and ferric compounds yield a black precipitate with ammonium sulfide TS. This precipitate is dissolved by cold dilute hydrochloric acid TS with evolution of hydrogen sulfide.

Magnesium

Solutions of magnesium salts in the presence of ammonium chloride yield no precipitate with ammonium carbonate TS, but a white, crystalline precipitate, which is insoluble in ammonia TS, is formed upon the subsequent addition of sodium phosphate TS.

Manganese

Solutions of manganese salts yield with ammonium sulfide TS a salmon-coloured precipitate which dissolves in acetic acid.

Nitrate

When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture cooled, and a solution of ferrous sulfate superimposed, a brown colour is produced at the junction of the two liquids. Brownish red fumes are evolved when a nitrate is heated with sulfuric acid and metallic copper. Nitrates do not decolourize acidified potassium permanganate TS (distinction from nitrites).

Nitrite

Nitrites yield brownish red fumes when treated with dilute mineral acids or acetic acid. A few drops of potassium iodide TS and a few drops of dilute sulfuric acid TS added to a solution of a nitrite liberate iodine which colours starch TS blue.

Peroxide

Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue colour upon the addition of potassium dichromate TS. On shaking the mixture with an equal volume of ether and allowing the liquids to separate, the blue colour is transferred to the ether layer.

Phosphate

Neutral solutions of orthophosphates yield with silver nitrate TS a yellow precipitate, which is soluble in dilute nitric acid TS or in ammonia TS. With ammonium molybdate TS, a yellow precipitate, which is soluble in ammonia TS, is formed.

Potassium

Potassium compounds impart a violet colour to a non-luminous flame if not masked by the presence of small quantities of sodium. In neutral, concentrated or moderately concentrated solutions of potassium salts, sodium bitartrate TS slowly produces a white, crystalline precipitate which is soluble in ammonia TS and in solutions of alkali hydroxides or carbonates. The precipitation may be accelerated by stirring or rubbing the inside of the test tube with a glass rod or by the addition of a small amount of glacial acetic acid or ethanol.

Sodium

Sodium compounds, after conversion to chloride or nitrate, yield with cobalt-uranyl acetate TS a golden-yellow precipitate, which forms after several min agitation. Sodium compounds impart an intense yellow colour to a non-luminous flame.

Sulfate

Solutions of sulfates yield with barium chloride TS a white precipitate which is insoluble in hydrochloric and nitric acids. Sulfates yield a white precipitate with lead acetate TS, which is

soluble in ammonium acetate solution. Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

Sulfite

When treated with dilute hydrochloric acid TS, sulfites and bisulfites yield sulfur dioxide, recognizable by its characteristic odour. This gas blackens filter paper moistened with mercurous nitrate TS.

Thiosulfate

Solutions of thiosulfates yield with hydrochloric acid a white precipitate which soon turns yellow, liberating sulfur dioxide, recognizable by its odour. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet colour which quickly disappears.

Zinc

Zinc salts, in the presence of sodium acetate, yield a white precipitate with hydrogen sulfide. This precipitate, which is insoluble in acetic acid, is dissolved by dilute hydrochloric acid TS. A similar precipitate is produced by ammonium sulfide TS in neutral or alkaline solutions. Solutions of zinc salts yield with potassium ferrocyanide TS a white precipitate which is insoluble in dilute hydrochloric acid TS.

Organic Ions and Compounds

Acetate

Acetic acid or acetates, when warmed with sulfuric acid and alcohol, form ethyl acetate, recognizable by its characteristic odour. With neutral solutions of acetates, ferric chloride TS produces a deep red colour which is destroyed by the addition of a mineral acid.

Alginate

Dissolve as completely as possible 0.01 g of the sample by shaking with 0.15 ml of 0.1 N sodium hydroxide and add 1 ml of acid ferric sulfate TS. Within 5 min, a cherry-red colour develops that finally becomes deep purple.

Ascorbate

To 2 ml of a 2% solution of the sample in water add 2 ml of water, 0.1 g of sodium bicarbonate and about 0.02 g of ferrous sulfate. Shake and allow to stand. A deep violet colour is produced, which disappears on the addition of 5 ml of dilute sulfuric acid TS.

Benzoate

Neutral solutions of benzoates yield a salmon-coloured precipitate with ferric chloride TS. From moderately concentrated solutions of benzoate, dilute sulfuric acid TS precipitates free benzoic acid, which is readily soluble in ether.

Citrate

When a few mg of a citrate are added to a mixture of 15 ml of pyridine and 5 ml of acetic anhydride, a carmine red colour is produced.

Gluconates

Dissolve a quantity of the sample in water to obtain a solution containing 10 mg/ml, heating in a water bath at 60°, if necessary. Similarly, prepare a standard solution of potassium gluconate in water containing 10 mg/ml.

Apply separate 5-µl portions of the test solution and the standard solution on a suitable thin-layer chromatographic plate coated with 0.25-mm layer of chromatographic silica gel, and allow to dry. Develop the chromatogram in a solvent system consisting of a mixture of ethanol, water, ammonium hydroxide, and ethyl acetate (50:30:10:10) until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 110° for 20 min. Allow to cool, and spray with a reagent, prepared as follows: Dissolve 2.5 g of ammonium molybdate in about 50 ml of 2 N sulfuric acid in a 100-ml volumetric flask, add 1.0 g of ceric sulfate, swirl to dissolve, dilute with 2 N sulfuric acid to volume, and mix. Heat the plate at 110° for about 10 min. The principal spot obtained from the test solution corresponds in colour, size, and retention to that obtained from the standard solution.

Glutamate

Proceed as directed under Thin Layer Chromatography (see Analytical Techniques) using the following conditions:

Sample: 1 µl of 1 in 100 solution of the sample. Add a few drops of ammonium hydroxide TS if required to dissolve.

Reference: 1 µl of a 1 in 100 solution of monosodium L-glutamate

Solvent: A mixture of 2 volumes of n-butanol, 1 volume of glacial acetic acid and 1 volume of water

Adsorbent: Silica gel

Stop the development when the solvent front has advanced about 10 cm from the point of application. Dry the plate at 80° for 30 min. Spray ninhydrin TS on the plate, heat at 80° for 10 min and observe the plate under natural light. The R_f value of the sample and that of the reference standard are identical.

Glycerol

Heat a few drops of the sample in a test tube with about 0.5 g of potassium bisulfate; pungent vapours of acrolein are evolved.

Lactate

When solutions of lactates are acidified with sulfuric acid, and potassium permanganate TS is added and the mixture heated, acetaldehyde, recognizable by its distinctive odour, is evolved.

Malate

Transfer the solution described in the individual monograph into a porcelain dish and add 10 mg of sulfanilic acid. Heat the solution on a water bath for a few min, add 5 ml of a 1 in 5 solution of sodium nitrite and heat slightly. Make alkaline with sodium hydroxide TS. A red colour is produced.

Organic Phosphate

To the solution given in the monograph add 2 ml of magnesia mixture TS. No precipitate is formed. Add 5 ml of nitric acid, boil for 10 min, neutralize with strong ammonia TS, add water to make to 100 ml, add ammonium molybdate TS, and warm. A yellow precipitate is formed, which dissolves in sodium hydroxide TS or ammonia TS.

Ribose

To 3 ml of a 3 in 10,000 solution of the sample in water, add 0.2 ml of a 1 in 10 solution of orcinol in ethanol and subsequently 3 ml of a 1 in 1,000 hydrochloric acid solution of ferric ammonium sulfate. Heat in a water bath for 10 min. A green colour is produced.

Tartrate

When a few mg of a tartrate are added to a mixture of 15 ml of pyridine and 5 ml of acetic anhydride, an emerald green colour is produced.

INORGANIC COMPONENTS

Acid-Insoluble Matter

Transfer 2 g of the sample, accurately weighed, into a 250-ml beaker containing 150 ml of water and 1.5 ml of sulfuric acid TS. Cover the beaker with a watch glass and heat the mixture on a steam bath for 6 h rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod and replacing any water lost by evaporation. Weigh 500 mg of a suitable acid washed filter aid, pre-dried at 105° for 1 h, to the nearest 0.1 mg, add this to the sample solution and filter through a tared Gooch crucible provided with an asbestos pad. Wash the residue several times with hot water, dry the crucible and its contents at 105° for 3 h, cool in a desiccator and weigh. The difference between the total weight and the weight of the filter aid plus crucible and pad is the weight of the Acid-insoluble matter. Calculate as percentage.

Arsenic Limit Test

Note1: *Method I referenced in older specifications has been deleted. The colourimetric procedure described in Method II may be used. However, it is recommended that, whenever possible, that the determination of arsenic be carried out using AAS-hydride technique/ICP method.*

Note 2: *Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine (Method II). Antimony, which forms stibine, is the only metal likely to produce a positive interference. Stibine forms a red coloured complex with silver diethyldithiocarbamate reagent which has a maximum absorbance at 510 nm. But at 535 - 540 nm the absorbance of the antimony complex is so diminished that the results of arsenic would not be affected significantly.*

Note 3: *All reagents used in the limit test for arsenic should be very low in arsenic content.*

Method II (Colourimetric Procedure)

Apparatus

The general apparatus is shown in Figure 1. It consists of a 125-ml arsine generator flask with a 24/40 standard-taper joint fitted with a scrubber unit and an absorber tube connected by a capillary of inside diameter 2 mm and outside diameter 8 mm via a ball-and-socket joint, secured with a No. 12 clamp, connecting the units. Alternatively, an apparatus embodying the principle of the general assembly described and illustrated may be used.

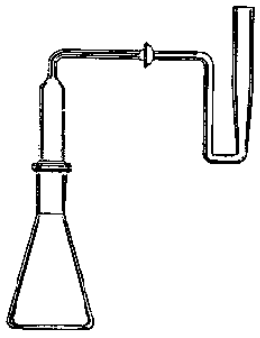


Figure 1. Apparatus for Arsenic Limit Test – Method II

Reagents

Silver Diethyldithiocarbamate Solution

Dissolve 1 g of recrystallized silver diethyldithiocarbamate, $(C_2H_5)_2NCSSAg$, in 200 ml of reagent grade pyridine in a fume hood. Store this solution in a light-resistant container and use within 1 month.

Silver diethyldithiocarbamate is available commercially or may be prepared as follows. Dissolve 1.7 g of reagent grade silver nitrate in 100 ml of water. In a separate container, dissolve 2.3 g of sodium diethyldithiocarbamate, $(C_2H_5)_2NCSSNa \cdot 3H_2O$, in 100 ml of water, and filter. Cool both solutions to about 15°, mix the two solutions, while stirring, collect the yellow precipitate in a medium-porosity sintered-glass crucible or funnel, and wash with about 200 ml of cold water.

Recrystallize the reagent, whether prepared as directed above or obtained commercially, as follows: Dissolve in freshly distilled pyridine, using about 100 ml of solvent for each g of reagent, and filter. Add an equal volume of cold water to the pyridine solution, while stirring. Filter off the precipitate, using suction, wash with cold water, and dry in vacuum at room temperature for 2 to 3 h. The dry salt is pure yellow in colour and should show no change in character after 1 month when stored in a light-resistant container. Discard any material that changes in colour or develops a strong odour.

Standard Arsenic Solution

Weigh accurately 132.0 mg of arsenic trioxide that has been finely pulverized and dried for 24 h over a suitable desiccant, and dissolve it in 5 ml of sodium hydroxide solution (1 in 5). Neutralize the solution with diluted sulfuric acid TS, add 10 ml in excess, and dilute to 1,000.0 ml with recently boiled water, and mix. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, add 10 ml of diluted sulfuric acid TS, dilute to volume with recently boiled water and mix.

Use this final solution, which contains 1 µg of arsenic (As) in each ml, within 3 days.

Stannous Chloride Solution

Dissolve 40 g of reagent grade stannous chloride dihydrate, $SnCl_2 \cdot 2H_2O$, in 100 ml of hydrochloric acid. Store the solution in a glass container and use within 3 months.

Lead Acetate-Impregnated Cotton

Soak cotton in a saturated solution of reagent grade lead acetate, squeeze out the excess solution, and dry in a vacuum at room temperature.

Note: *When preparing and using the cotton, take great care to avoid lead contamination.*

Preparation of the Sample Solution

The solution obtained by treating the sample as directed in an individual monograph is used directly as the Sample Solution in the Procedure. Sample solutions of organic compounds are prepared in the generator flask (Figure 3), unless otherwise directed, according to the following general procedure:

Caution: *Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Appropriate safety precautions must be employed at all times.*

Note: *If halogen-containing compounds are present, use a lower temperature while heating the sample with sulfuric acid, do not boil the mixture, and add the peroxide, with caution, before charring begins, to prevent loss of trivalent arsenic.*

Transfer 1.0 g of the sample into the generator flask, add 5 ml of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° on a hot plate in a fume hood until charring begins. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 ml.) After the sample has been initially decomposed by the acid, add with caution, dropwise, 30% hydrogen peroxide, allowing the reaction to subside and reheating between drops. The first few drops must be added very slowly with sufficient mixing to prevent a rapid reaction, and heating should be discontinued if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls or bottom of the flask during digestion. Maintain oxidizing conditions at all times during the digestion by adding small quantities of the peroxide whenever the mixture turns brown or darkens. Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250° - 300° until fumes of sulfuric acid are copiously evolved, and the solution becomes colourless, or retains only a slight straw colour.

Cool, add cautiously 10 ml of water, again evaporate (fumes of sulfuric acid evolved), and cool. Add cautiously 10 ml of water, mix, wash the sides of the flask with a few ml of water, and dilute to 35 ml.

Procedure

If the sample solution was not prepared in the generator flask, transfer to the flask a volume of the solution, prepared as directed, equivalent to 1.0 g of the substance being tested and add water to make 35 ml.

Add 20 ml of dilute sulfuric acid (1 in 5), 2 ml of potassium iodide TS, and 0.5 ml of *Stannous Chloride Solution*, and mix. Allow the mixture to stand for 30 min at room temperature. Pack the scrubber tube with two plugs of Lead Acetate-Impregnated Cotton, leaving a small air space between the two plugs, lubricate the ground-glass joints with stopcock grease, if necessary, and connect the scrubber unit with the absorber tube. Transfer 3.0 ml of silver diethyldithiocarbamate solution to the absorber tube, add 3.0 g of granular zinc (20-mesh) to the mixture in the flask, and immediately insert the standard-taper joint in the flask. Allow the evolution of arsine and colour development to proceed at room temperature (25 ± 3°) for 45 min, swirling the flask gently at 10-min intervals. (The addition of a small amount of isopropanol to the generator flask may improve the uniformity of the rate of gas evolution.) Disconnect the absorber tube from the generator and scrubber units, and transfer the Silver diethyldithiocarbamate solution to a 1-cm absorption cell. Determine the absorbance at the wavelength of maximum absorption between 535 nm and 540 nm with a suitable spectrophotometer or colorimeter, using Silver diethyldithiocarbamate solution as the blank. The absorbance due to any red colour from the solution of the sample does not exceed that produced by 3.0 ml of standard arsenic solution (3 µg As) when treated in the same manner and under the same conditions as the sample. The room temperature during the generation of arsine from the standard should be held to within ± 2° of that observed during the determination of the sample.

Ash

Ash (Total)

Accurately weigh a known quantity of sample (depending on the ash content such that about 20 mg of ash is obtained) in a tared crucible, ignite at a low temperature (about 550°), not to exceed a very dull redness, until free from carbon, cool in a desiccator, and weigh. If a carbon-free ash is not obtained, wet the charred mass with hot water, spread the residue using

a glass rod, dry it in an air oven and reignite. If a carbon-free ash is still not obtained, cool the crucible, add 15 ml of ethanol, break up the ash with a glass rod, then burn off the ethanol, again heat the whole to dull redness, cool in a desiccator, and weigh.

Note: *If difficulty with oxidizing organic material is found, the use of an ash aid such as ammonium nitrate may prove to be more satisfactory. Addition of a few drops of hydrogen peroxide facilitate oxidation of organic matter.*

Ash (Acid-insoluble)

Boil the ash obtained as directed under Ash (Total) above, with 25 ml of dilute hydrochloric acid TS for 5 min, collect the insoluble matter on a suitable ash-less filter, wash with hot water, ignite at 800 ± 25°, cool, and weigh. Calculate the percentage of acid-insoluble ash from the weight of the sample taken.

Ash (Sulfated ash)

Method I (for solids)

Transfer the quantity of the sample directed in the individual monograph to a tared 50- to 100-ml platinum dish or other suitable container. Add sufficient diluted sulfuric acid TS to moisten the entire sample. Heat gently, using a hot plate, an Argand burner, or an infrared heat lamp, until the sample is dry and thoroughly charred, then continue heating until all of the sample has been volatilized or nearly all of the carbon has been oxidized, and cool. Moisten the residue with 0.5 ml of sulfuric acid TS, and heat in the same manner until the remainder of the sample and any excess sulfuric acid have been volatilized. Finally ignite in a muffle furnace at 800 ± 25° for 15 min or longer, if necessary, to complete ignition, cool in a desiccator, and weigh.

(**Note:** *In order to promote volatilization of sulfuric acid, it is advisable to add a few pieces of ammonium carbonate just before completing ignition*.)

Method II (for liquids)

Unless otherwise directed, transfer the required weight of the sample to a suitable tared container, add 10 ml of diluted sulfuric acid TS, and mix thoroughly. Evaporate the sample completely by heating gently without boiling, and cool. Finally, ignite in a muffle furnace at 800 ± 25° for 15 min or longer, cool in a desiccator, and weigh.

Chlorides Limit Test

Unless otherwise specified, place the prescribed quantity of the sample in a Nessler tube, dissolve it in about 30 ml of water, and neutralize with dilute nitric acid TS if the solution is alkaline. Add 6 ml of dilute nitric acid TS and dilute to 50 ml with water. If the use of a sample solution is prescribed, transfer the sample solution into a Nessler tube and dilute to 50 ml with water. Transfer the prescribed volume of 0.01 N hydrochloric acid into another Nessler tube to serve as the standard, add 6 ml of dilute nitric acid TS, and dilute to 50 ml with water.

If the solution containing the sample is not clear, filter both solutions under the same conditions. Add 1 ml of silver nitrate TS to each solution, mix thoroughly, and allow to stand for 5 min protected from direct sunlight. Compare the turbidity of the two solutions by observing the Nessler tubes from the sides and the tops against a black background. The turbidity of the sample solution does not exceed that of the standard.

Chromium Limit Test

Note: *The limit test described below is designed to show whether the sample contains more or less than 20 mg/kg of chromium. It is recommended to use an appropriate AAS/ICP method in the place of the limit test, if possible, for quantitative determination.*

Procedure

Weigh 1.0 g of the sample into a quartz dish. Char the material, raising the temperature slowly. Allow to cool, add 10 ml of a 25% magnesium nitrate solution; evaporate, heating slowly until no more nitrous vapour evolves. Heat the material in an oven at 600° until all black particles have disappeared (1 h).

Dissolve the residue by adding 10 ml of 4 N sulfuric acid and 20 ml of water. Heat on a water bath for about 5 min.

Add 0.5 ml of 0.1 N potassium permanganate. Add more permanganate if the solution decolourizes. Cover with a watch glass and heat on a water bath for about 20 min. Add 5% sodium azide solution, one drop every 10 sec, until the excess potassium permanganate has been removed (avoid excess of sodium azide; 2 drops are usually sufficient). Cool the solution in running water, and filter if manganese dioxide (black precipitate) is evident. Transfer the solution to a 50-ml volumetric flask. Add 2.5 ml of 5 M sodium dihydrogenphosphate, add 2 ml of diphenyl carbazide TS and fill to the mark with water. Measure the absorbance at 540 nm 30 min after adding the diphenyl carbazide TS. A blank with the latter two reagents should show no colour or only a slight purple colour.

At the same time run a parallel test with 1.00 ml of standard chromate TS (1 ml = 20 µg Cr) and a few ml of saccharose placed into a second quartz dish. Treat the mixture exactly as the sample and measure the extinction at the same wavelength.

Calculate the chromium content of the sample from the two extinction values observed.

Cyclic Phosphate Determination

Note: *The method uses perchloric acid in one of the reagents. Special care shall be taken while handling it and all operations shall be conducted in a perchloric acid fume cup board.*

Principle

The method is based on two-dimensional paper chromatography, in which the development is first carried out in one direction using a basic solvent. The paper is then turned through 90° and chromatographed using an acidic solvent. Spots are revealed by spraying with a perchloric acid/molybdate reagent, and are identified and qualitatively assembled by reference to chromatograms of standard phosphates. Quantitative estimation is effected by cutting out the 'spots', washing the paper with ammonia, subsequent determination of the phosphorus content by colourimetry of the molybdenum blue complex and calculation of cyclic phosphate content as % $NaPO_3$.

Reagents

Solvent A (basic): Mix together: 400 ml isopropanol, 200 ml isobutanol, 300 ml deionised water and 10 ml 0.880 sp.gr. ammonia solution.

Solvent B (acidic): Mix together: 750 ml isopropanol and 250 ml deionised water. Add: 50 g trichloro-acetic acid and 2.5 ml 0.880 sp.gr. ammonia solution.

Spray reagent: To 50 ml deionised water, add: 5 ml 60% perchloric acid, 1 ml conc. HC1 (1.18 sp.gr.) and 1 g ammonium molybdate. Make up to 100 ml with deionised water

Standard Phosphate Solutions: Prepare standard solutions of sodium tri, tetra, hexa, and octameta phosphates containing 2 µg/µl (0.2% w/v).

Procedure

Draw faint pencil lines 2.5 cm from the bottom edge and 2.5 cm from the right-hand side of a 23 x 23 cm square piece of the chromatography paper. Apply 1 µl of a 10% w/v solution of the sample at the intersection of the two pencil lines. Allow the paper to dry, curve it into a cylinder, and secure with plastic clips. Stand the cylinder in the tank containing the basic solvent (Solution A), the immersion depth being about 6 mm and allow the solvent front to rise to a height of 20 cm. Remove the paper from the tank and mark the position of the solvent front. Dry the paper in an air oven at 50° and cut off the excess paper above the solvent front.

Develop the paper in acid solvent (Solution B), with the previous right-hand edge to the bottom of the cylinder, until the solvent front has travelled 20 cm. Remove and dry the paper and spray with the acid ammonium molybdate solution. Develop the spots produced by placing the paper under the U.V. lamp at 250 nm for a few minutes.

Mark out a separate piece of chromatography paper as described above. At the intersection of the pencil lines apply 1 µl of each of the meta phosphate standard solutions in turn, drying the paper after each application. Treat this standard paper in a similar manner to that described for the sample. Both tests must be run concurrently using the same solvents, tanks and spray.

Compare the sample and standard chromatograms, and identify the 'spots' with the aid of the R_f values given in the Table below.

Phosphate	**R_f basic**	**R_f acidic**
Tri-meta	0.49	0.13
Tetra-meta	0.36	0.05
Hexa-meta	0.27	0.02
Octa-meta	0.21	0.01
Ortho	0.32	0.71
Pyro	0.26	0.40

R_f values for ortho-, pyro- and cyclic phosphates
(Values should be taken as a guide only).

If a spot of particular interest is too weak, the chromatogram should be repeated using 2 or 5 µl sample solution instead of 1 µl. About 2 µl of each of the various phosphates should be visible.

An approximation of the quantities of each component in the sample will be gained by a visual comparison of the two chromatograms. For a more accurate measurement, cut out each spot and analyze for total phosphorus by the following method.:

Soak each cut out area of chromatography paper in 25.00- ml of 0.1 N ammonium hydroxide solution for at least 1 h. Pipet a 20.00- ml aliquot of the resulting solution into a 50 -ml volumetric flask, add 5 ml of 10 N sulfuric acid and heat in a boiling water bath for 30 min

to hydrolyse the cyclic phosphates to orthophosphate. Cool to room temperature, add 1 ml of 12.5% ammonium molybdate solution, shake the flask and then add 1 ml of 0.6% hydrazine hydrochloride. Make up to volume with water and place the flask in a boiling water bath for exactly 10 min. Cool rapidly in a cold water bath and measure the absorbance of the solution in a spectrophotometer at 830 nm using distilled water as the reference solution. Perform a blank determination using an equal area of chromatography paper known not to include any phosphate spots and subtract the blank value from the test values. Determine the amount of phosphorus present by reference to a calibration curve of absorbance at 830 nm obtained using samples of standard amounts of potassium dihydrogen orthophosphate.

Where a spot is ill-defined, compare with the standard chromatogram and cut out the zone where the spot should appear. Cut out the area occupied by all the metaphosphates to obtain total cyclic content.

Calculation

If x = is µl of a 10% solution of sample put on paper and y is= µg P obtained by attached method, then:

%P in sample = y / x

% cyclic phosphates expressed as $NaPO_3$= (102 /31)(y / x)

Fluoride Limit Test

Method I - Thorium Nitrate Colorimetric Method

This method should be used unless otherwise directed in the individual monograph.

Caution: *When applying this test to organic compounds, the temperature at which the distillation is conducted must be rigidly controlled at all times to the recommended range of 135° to 140° to avoid the possibility of explosion.*

Note: *To minimize the distillation blank resulting from fluoride leached from the glassware, the distillation apparatus should be treated as follows:*

Treat the glassware with hot 10% sodium hydroxide solution, followed by flushing with tap water and rinsing with distilled water. At least once daily, treat in addition by boiling down 15 to 20 ml of dilute sulfuric acid (1 in 2) until the still is filled with fumes; cool, pour off the acid, treat again with 10% sodium hydroxide solution, and rinse thoroughly. For further details, see sections 25.050 and 25.054 in Official Methods of Analysis of the AOAC, Thirteenth Edition, 1980.

Unless otherwise directed, place a 5.0 g sample and 30 ml of water in a 125 ml distillation flask having a side arm and trap. The flask is connected with a condenser and carries a thermometer and a capillary tube, both of which must extend into the liquid. Slowly add, with continuous stirring, 10 ml of perchloric acid, and then add 2 or 3 drops of silver nitrate solution (1 in 2) and a few glass beads. Connect a small dropping funnel or a steam generator to the capillary tube. Support the flask on an asbestos mat with a hole that exposes about one third of the flask to the flame. Distil until the temperature reaches 135°. Add water from the funnel or introduce steam through the capillary, maintaining the temperature between 135° and 140° at all times. Continue the distillation until 100 ml of distillate has been collected. After the 100 ml portion (Distillate A) is collected, collect an additional 50 ml portion of distillate (Distillate B) to ensure that all of the fluorine has been volatilized.

Place 50 ml of Distillate A in a 50 ml Nessler tube. In another similar Nessler tube place 50 ml of water distilled through the apparatus as a control. Add to each tube 0.1 ml of a filtered solution of sodium alizarinsulfonate (1 in 1,000) and 1 ml of freshly prepared hydroxylamine hydrochloride solution (1 in 4,000), and mix well. Add, dropwise and with stirring, either 1 N or 0.05 N sodium hydroxide, depending upon the expected volume of volatile acid distilling over, to the tube containing the distillate until its colour just matches that of the control, which is faintly pink. Then add to each tube 1.0 ml of 0.1 N hydrochloric acid, and mix well. From a burette, graduated in 0.05 ml, add slowly to the tube containing the distillate enough thorium nitrate solution (1 in 4,000) so that, after mixing, the colour of the liquid just changes to a faint pink. Note the volume of the solution added, then add exactly the same volume to the control, and mix. Now add to the control solution, sodium fluoride TS (10 µg F per ml) from a burette to make the colours of the two tubes match after dilution to the same volume. Mix well, and allow all air bubbles to escape before making the final colour comparison. Check the endpoint by adding 1 or 2 drops of sodium fluoride TS to the control. A distinct change in colour should take place. Note the volume of sodium fluoride TS added.

Dilute Distillate B to 100 ml, and mix well. Place 50 ml of this solution in a 50 ml Nessler tube, and follow the procedure used above for Distillate A. The total volume of sodium fluoride TS required for the solutions from both Distillate A and Distillate B should not exceed 2.5 ml.

Method II - Ion-Selective Electrode Method A

Reagents

Buffer Solution: Dissolve 36 g of cyclohexylenedinitrilotetraacetic acid (CDTA) in sufficient 1 M sodium hydroxide to make 200 ml. Transfer 20 ml of this solution (equivalent to 4 g of disodium CDTA) into a 1,000-ml beaker containing 500 ml of water, 57 ml of glacial acetic acid, and 58 g of sodium chloride, and stir to dissolve. Adjust the pH of the solution to between 5.0 and 5.5 by the addition of 5 M sodium hydroxide, then cool to room temperature, dilute to 1,000 ml with water, and mix.

Procedure

Unless otherwise directed in the individual monograph, transfer 8.0 g of sample and 20 ml of water to a 250-ml distilling flask, cautiously add 20 ml of perchloric acid, and then add 2 or 3 drops of silver nitrate solution (1 in 2) and a few glass beads. Following the directions, and observing the **Caution** and **Note**, as given under Method I, distil the solution until 200 ml of distillate has been collected.

Treat the glassware with hot 10% sodium hydroxide solution, followed by flushing with tap water and rinsing with distilled water. At least once daily, treat in addition by boiling down 15 to 20 ml of dilute sulfuric acid (1 in 2) until the still is filled with fumes; cool, pour off the acid, treat again with 10% sodium hydroxide solution, and rinse thoroughly. For further details, see sections 25.050 and 25.054 in Official Methods of Analysis of the AOAC, Thirteenth Edition, 1980.

Transfer a 25.0-ml aliquot of the distillate into a 250-ml plastic beaker, and dilute to 100 ml with the Buffer Solution. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) of a suitable ion-selective electrode apparatus in the solution. Adjust the calibration control until the indicator needle points to the center of the logarithmic concentration scale, allowing sufficient time for equilibration (about 20 min) and stirring constantly during the equilibration period and throughout the remainder of the procedure. Pipet 1.0 ml of a solution containing 100 µg of fluoride (F) ion per ml (prepared by

dissolving 22.2 mg of sodium fluoride, previously dried at 200° for 4 h, in sufficient water to make 100.0 ml) into the beaker, allow the electrode to come to equilibrium, and record the final reading on the logarithmic concentration scale. (**Note:** *Follow the instrument manufacturer's instructions regarding precautions and interferences, electrode filling and check, temperature compensation, and calibration*.)

Calculation

Calculate the fluoride content, in mg/kg of the sample taken by the formula

$$[IA/(R - I)] \times 100 \times [200/25W]$$

in which I is the initial scale reading before the addition of the sodium fluoride solution; A is the concentration, in µg per ml, of fluoride in the sodium fluoride solution added to the sample solution; R is the final scale reading, after addition of the sodium fluoride solution; and W is the original weight of the sample in g.

Method III - Ion-Selective Electrode Method B

Reagents

Sodium Fluoride Solution (5 µg F per ml): Transfer 2.210 g of sodium fluoride, previously dried at 200° for 4 h and accurately weighed, into a 400-ml plastic beaker, add 200 ml of water, and stir until dissolved. Quantitatively transfer this solution into a 1,000-ml volumetric flask with the aid of water, dilute to volume with water, and mix. Store this stock solution in a plastic bottle. On the day of use, transfer 5.0 ml of the stock solution into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

Standard Curve: Transfer into separate 250-ml plastic beakers 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 ml of the Sodium Fluoride Solution, add 50 ml of water, 5 ml of 1 N hydrochloric acid, 10 ml of 1 M sodium citrate, and 10 ml of 0.2 M disodium EDTA to each beaker, and mix. Transfer each solution into separate 100-ml volumetric flasks, dilute to volume with water, and mix. Transfer a 50-ml portion of each solution into separate 125-ml plastic beakers, and measure the potential of each solution with a suitable ion-selective electrode, using a suitable reference electrode. Plot the standard curve on two-cycle semi-logarithmic paper with µg F per 100 ml solution on the logarithmic scale.

Procedure

Transfer 1.00 g of the sample into a 150-ml glass beaker, add 10 ml of water, and while stirring continuously, add 20 ml of 1 N hydrochloric acid slowly to dissolve the sample. Boil rapidly for 1 min, then transfer into a 250-ml plastic beaker, and cool rapidly in ice water. Add 15 ml of 1 M sodium citrate and 10 ml of 0.2 M disodium EDTA, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 M sodium hydroxide, if necessary, then transfer into a 100-ml volumetric flask, dilute to volume with water, and mix. Transfer a 50-ml portion of this solution into a 125-ml plastic beaker and measure the potential of the solution with the apparatus described under Standard Curve. Determine the fluoride content, in µg, of the sample from the Standard Curve.

Method IV - Ion-Selective Electrode Method C

Reagents

Buffer Solution: Dissolve 150 g of sodium citrate dehydrate and 10.3 g of disodium EDTA dihydrate in 800 ml of water, adjust the pH to 8.0 with 50% sodium hydroxide solution, and dilute to 1000 ml with water.

Fluoride Standard Solutions

- 1000 mg/kg Fluoride Standard: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-ml volumetric flask and dissolve in and dilute to volume with water. The resulting solution contains 1000 mg of fluoride per milliliter.
- 50 mg/kg Fluoride Standard: Pipet 50 ml of the 1000 mg/kg Fluoride Standard into a 1000-ml volumetric flask. Dilute to volume with water.
- 10 mg/kg Fluoride Standard: Pipet 100 ml of the 50 mg/kg Fluoride Standard into a 500-ml volumetric flask. Dilute to volume with water.

Fluoride Limit Solutions (for a 1-g sample)

- 50 mg/kg Fluoride Limit Solution (1 mg/kg fluoride standard): Pipet 50 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.
- 10 mg/kg Fluoride Limit Solution (0.2 mg/kg fluoride standard): Pipet 10 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.

Fluoride Limit Solutions (for a 2-g sample)

- 50 mg/kg Fluoride Limit Solution (2 mg/kg fluoride standard): Pipet 100 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.
- 10 mg/kg Fluoride Limit Solution (0.4 mg/kg fluoride standard): Pipet 20 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.

Note: *Store all standard and limit solutions in plastic containers.*

Sample Preparation: Accurately weigh the amount of sample specified in the monograph, transfer it into a 100-ml volumetric flask, and dissolve it in a minimal amount of water. Add 50.0 ml of the Buffer Solution, dilute to volume with water, and mix.

Electrode Calibration: Pipet 50 ml of the Buffer Solution into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker and stir. At 5-min intervals, add 100 µl and 1000 µl of the 1000 mg/kg Fluoride Standard and read the potential, in millivolts, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 54 to 60 mV at 25°. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions.

Procedure

Transfer the entire sample into a plastic beaker. Place the electrode into the beaker, allow the solution to equilibrate for 5 min with stirring, and read the potential, in millivolts. Remove and rinse the electrode(s) with water. In another beaker, using a pipet, add 50 ml of the Buffer Solution followed by 50 ml of the Fluoride Limit Solution that best reflects the fluoride limit of the sample. Place the electrode in the beaker, equilibrate for 3 min, and read the potential

in millivolts. If the potential of the Fluoride Limit Solution is less than that of the sample, the sample passes the test criterion for maximum acceptable fluoride level limit.

Iron Limit Test

Note: *It is recommended to use an appropriate AAS/ICP method in the place of the limit test, if possible, for quantitative determination of iron.*

To 0.5 g of the sample, weighed to the nearest mg, add 2 ml of hydrochloric acid, and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml of hydrochloric acid and 20 ml of water, and add a few drops of bromine TS. Boil the solution in a fume hood to remove the bromine, cool, dilute with water to 25 ml, and then add 50 mg of ammonium persulfate and 5 ml of ammonium thiocyanate TS. Any red colour produced should not exceed that of a control solution made the same way as the test solution but containing instead of the sample the amount of Iron Standard TS prescribed in the individual monograph.

Loss on Drying

Loss on drying is the amount of volatile matter expelled under the conditions specified in the monograph. Because the volatile matter may include materials other than water, this test is designed for compounds in which the loss on drying may not definitely be attributable to water alone. The water content may be determined by a method such as *Karl Fischer* titration method

Note: *Suitable precautionary steps should be taken when weighing hygroscopic or deliquescent samples to ensure that they do not absorb moisture.*

Unless otherwise directed in the individual monograph, conduct the determination on 1 to 2 g of the substance, previously well mixed and accurately weighed. Reduce the sample to a fine powder when it occurs as crystals. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 min under the same conditions as will be employed in the determination. Transfer the sample into the bottle, replace the cover, and weigh the bottle and the sample. Distribute the sample as evenly as practicable to a depth of about 5 mm, and not over 10 mm in the case of bulky materials. Place the bottle with its contents in the drying chamber, removing the stopper and leaving it also in the chamber, and dry the sample at the temperature and for the time specified in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of *Loss on Drying*, prepare the sample as described above, then place it in a vacuum desiccator containing sulfuric acid. Evacuate the desiccator to 130 Pa (1 mm of mercury), maintain this vacuum for 24 h, and then weigh the dried sample.

Loss on Ignition

Proceed as directed for Loss on Drying. However, unless otherwise directed, ignite the sample at a temperature of 450 - 550° and use a platinum, quartz or porcelain dish instead of the weighing bottle.

Metallic Impurities

All the procedures for trace metals commence with dissolution of the sample and, if applicable, with destruction of organic matter in the sample. The trace metal content may then be determined by instrumental or chemical methods.

Atomic spectroscopy (atomic absorption as well as atomic emission) combines speed with accuracy and is widely used for the determination of metallic impurities.

Chemical methods depend on the formation of coloured compounds (complexes) with metal impurities under controlled conditions. The colour intensities of sample and standards are then compared visually or by using a spectrophotometer. Some of these methods lack in specificity and are subject to interferences from other trace elements.

Instrumental Methods

Principle

The samples are dissolved in acid or digested in a mixture of sulfuric, nitric and, in some cases perchloric acids. Metals (barium, cadmium, lead, copper, chromium, and zinc) in solution are determined by suitable atomic absorption spectrophotometry (AAS) or inductively coupled plasma (ICP) methods. The choice of flame/furnace AAS or ICP methods depend on the concentration of the analyte in the prepared sample solution (its concentration in the sample and limitations associated with the sample preparation). Furnace technique, offers better sensitivity, may be preferred over flame technique, when dealing with low levels of impurities in complex matrices. Antimony and arsenic may be determined by using a hydride generation AAS or ICP. Alternatively, antimony may be determined by flame atomic absorption but the hydride generation technique is more sensitive.

General precautions

Because of the minute amounts of metals involved special care must be taken to reduce the reagent blanks to as low a value as possible. Contamination in the laboratory is a major concern in trace metal analysis. All apparatus should be thoroughly cleaned with a mixture of hot dilute acids (1 part hydrochloric acid, 1 part concentrated nitric acid, and 3 parts water) followed by thorough washing with water immediately before use. All operations involving acids shall be carried out in the specified fume cupboards. **Note:** *Special care must be taken while using perchloric acid.*

Apparatus

Kjeldahl flasks, of silica or borosilicate glass (nominal capacity 100 ml) fitted with an extension to the neck by means of a B24 ground joint, as shown in Figure 2. The extension serves to condense the fumes and carries a tap funnel through which the reagents are introduced.

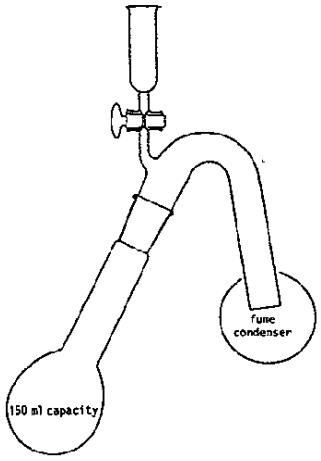


Figure 2. Modified Kjeldahl Flask (open type)

Atomic absorption spectrophotometer: Any commercial instrument operating in the absorption mode may be used providing it has required accessories (furnace and vapour generation) and facilities for the selection of the required oxidant/fuel combination from a choice of air, argon, nitrous oxide, hydrogen and acetylene and has a wavelength range from 180 to 600 nm.

All automated instruments have the facility of instrument control (selection of lamp, pre-warm up of lamp, wavelength and slit width and optimization) data acquisition and processing through a suitable software in a data station. However, with classical instruments, these need to be set manually and for operations in emission mode and measurements of absorption involving the generation of a gaseous hydride, a potentiometric recorder is necessary, preferably a multi-range type covering the range 1-20 mV.

Inductively coupled plasma–atomic emission spectrophotometer: Any commercial instrument, sequential or simultaneous system, operating in axial or radial mode may be used.

Reagents

Reagents shall be of an order of purity higher than accepted analytical reagent grade quality, preferably atomic spectroscopy grade. Metal-free water (Distilled water may be re-distilled from an all-glass apparatus or may be passed down a column of cation exchange resin, e.g., Amberlite IR 120(H), shall be used throughout. Deionized water (water subjected to reversed osmosis followed by passing ultra high quality deionisers) may also be used.

Standards

Preparations of atomic absorption standard solutions from pure metals or salts in the laboratory is cumbersome and subject to errors as large numbers of dilutions are involved. Single as well as mixed standard solutions in different concentration ranges are commercially available. Certified standards are also available for reference purpose. It is recommended to use commercially available standard solutions. The analyst must exercise proper care when diluting the stock standard solutions to not exceed a dilution factor of 20 in each step while diluting the concentrated solutions, in order to minimize dilution errors. Dilute the single standard stock solutions with 1% nitric acid to get the following solutions:

(a) Standard copper solution: 50 µg/ml

(b) Standard zinc solution: 10 µg/ml

(c) Standard chromium solution: 50 µg/ml

(d) Standard antimony solution: 200 µg/ml

(e) Standard lead solution: 100 µg/ml

(f) Standard barium solution: 200 µg/ml

(g) Standard arsenic solution: 5 µg/ml

(h) Standard cadmium solution: 10 µg/ml

Preparation of test solutions

Method I is applicable to substances soluble in dilute acids or mixtures of acids. Method II is used for other substances. The choice of method for the pre-treatment of a substance can also follow that given in the individual monograph.

Method I

Accurately weigh about 2.5 g of the sample and dissolve in a mixture of 4 ml of sulfuric acid and 5 ml of hydrochloric acid. Transfer the solution to a 50-ml volumetric flask. If barium is to be measured from the solution, add 0.0954 g of potassium chloride. Dilute to the mark with water. Mark this as Solution A.

Method II

Note: *Special care shall be taken while handling perchloric acid. All operations shall be carried out in a perchloric acid fume cupboard.*

Accurately weigh a known quantity of sample (about 2.5 g or based on the expected concentration of metal(s) in solution when made up to the volume, such that the concentration in solution will be higher than the first standard of the standard curve) into a 100 – 150-ml Kjeldahl flask, and add 5 ml of the dilute nitric acid. As soon as any initial reaction subsides, heat gently until further vigorous reactions cease and then cool. Add gradually 4 ml of sulfuric acid TS at such a rate as not to cause excessive frothing on heating (5-10 min is usually required) and then heat until the liquid darkens appreciably in colour, i.e., begins to char.

Add concentrated nitric acid slowly in small portions, heating between additions until darkening again takes place. Do not heat so strongly that charring is excessive, or loss of arsenic may occur; a small but not excessive amount of free nitric acid should be present throughout. Continue this treatment until the solution is only pale yellow in colour and fails to darken in colour on prolonged heating. If the solution is still coloured add 0.5 ml of the perchloric acid solution and a little concentrated nitric acid and heat for about 15 min, then add a further 0.5 ml of the perchloric acid solution and heat for a few minutes longer. Note the total amount of concentrated nitric acid used. Allow to cool somewhat and dilute with 10 ml of water. The solution should be quite colourless (if much iron is present it may be faintly yellow). Boil down gently, taking care to avoid bumping, until white fumes appear. Allow to cool, add a further 5 ml of water and again boil down gently to fuming. Finally, cool, add 10 ml 5 N hydrochloric acid and boil gently for a few minutes. Cool and transfer the solution to a 50-ml volumetric flask washing out the Kjeldahl flask with small portions of water. Add the washings to the graduated flask and dilute to the mark with water. If barium is to be measured

from the solution, add 0.0954 g of potassium chloride before dilution, as an ionizing buffer to prevent ionization of barium. Mark this as Solution A.

Prepare a reagent blank using the same quantities of reagents as used in the sample preparation.

Measurement of Antimony, Barium, Cadmium, Chromium, Copper, Lead and Zinc by Atomic Absorption Flame Technique

Preparation of standard curve solutions

To a series of 100-ml volumetric flasks pipette 0, 1, 2, 3, 4 and 5 ml of the appropriate standard solution [standards (a) to (f) and (h)] and dilute to about 50 ml. Add 8 ml concentrated sulfuric acid and 10 ml concentrated hydrochloric acid. In the case of barium [standard (f)], add 0.191 g of potassium chloride as an ionization buffer and shake to dissolve. Dilute to the mark with metal free water.

These solutions then contain 0, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/ml for lead; 0, 2.0, 4.0, 6.0, 8.0 and 10.0 µg/ml for barium and antimony; , 0, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml of cadmium and zinc or 0, 0.50, 1.0, 1.5, 2.0, 2.5 µg/ml for copper and chromium.

Instrumental conditions

Select the wavelengths and gases to be used for the particular element under consideration from the table below.

Element	Wave length (nm)	Gases
Antimony	217.6	Air/acetylene
Barium	553.6	Nitrous oxide/acetylene
Cadmium	228.8	Air/acetylene
Chromium	357.9	Nitrous oxide/acetylene
Copper	324.8	Air/acetylene
Lead	283.3	Air/acetylene
Zinc	213.9	Air/acetylene

The recommended settings for the various instrumental parameters vary from model to model, and certain parameters require optimization at the time of use to obtain the best results. Instruments should therefore be adjusted as described in the manufacturer's instructions using the type of flame and wavelength settings specified above.

Procedure

Set the atomic absorption spectrophotometer to the appropriate conditions. Aspirate the strongest standard containing the element to be determined and optimize the instrument settings to give full-scale or maximum deflection on the chart recorder. Measure the absorbances of the other standards and plot a graph showing the net absorbance against the concentration of the element in the standard solutions. Aspirate the solution A obtained from dissolution or the wet oxidation of the sample and the corresponding blank solution and

determine the net absorbance. If the concentration of the element in the solution is beyond the standard curve, dilute the solution as required and read the absorbance again. Using the graph prepared above, determine the concentration of the element in the sample solution.

Calculation

Element in the sample [mg/kg] =
[Concentration of element (µg/ml) x 50] / [Weight of sample taken (g)]

For certain elements flame atomic absorption method will not reach the required determination limits (e.g. a monograph specification limit for Pb of 1.0 mg/kg: A maximum of 5.0 grams of sample digested and made up to 50 ml will give a concentration of 0.1 µg/ml in solution which cannot be read by flame technique). The analyst may choose to use an electro-thermal atomization method under such circumstances.

Measurement of Antimony, Barium, Cadmium, Chromium, Copper, Lead and Zinc by Inductively Coupled Plasma (ICP) Technique

Preparation of standard curve solutions

The standard curve solutions given below are nominal in nature. The concentration of standard curve solutions differ based upon the operation mode of the torch (axial or radial) of the ICP instrument. The analyst may alternatively prepare appropriate standard curve solutions following the instrument operation manual.

To a series of 100-ml volumetric flasks pipette 0, 1, 2, 3, 4 and 5 ml of the appropriate standard solution [standards (a) to (h)] and dilute to about 50 ml. Add 8 ml concentrated sulfuric acid and 10 ml concentrated hydrochloric acid. Dilute to the mark with metal free water. These solutions then contain 0, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/ml for lead; 0, 2.0, 4.0, 6.0, 8.0 and 10.0 µg/ml for barium and antimony; , 0, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml of cadmium and zinc or 0, 0.50, 1.0, 1.5, 2.0, 2.5 µg/ml for copper and chromium.

Instrumental conditions

Select appropriate emission wavelengths to be used with each element under consideration. The recommended settings for the various instrumental parameters vary from model to model, and certain parameters require optimization at the time of use to obtain the best results. Instruments should therefore be adjusted as described in the manufacturer's instructions.

Procedure

Set the ICP instrument as stated in the operation manual. Activate the method and key in the standards data into the data station of the ICP. Aspirate the blank solution and set the instrument to zero, aspirate the standards and determine a standard curve for each element with emission intensity plotted against the concentration of the element in the standard solutions. Aspirate the solution A obtained from dissolution or the wet oxidation of the sample. If the concentration of the element in the solution is beyond the standard curve, dilute the solution as required and read it again. Using the standard curve, determine the element in the sample.

Calculation

Element in the sample [mg/kg] =
[Concentration of element (µg/ml) x 50] / [Weight of sample taken (g)]

Measurement of Lead and Cadmium by Atomic Absorption Electro-thermal Atomization (Furnace Atomization) Technique

Chemical modifier solutions:

Use of chemical modifier solutions in the furnace atomization allows use of higher ashing temperatures to reduce the background absorbance. These solutions must be of very high purity and are available commercially. One or more of the following modifiers may be used for the determination of lead and cadmium in different food additives.

- Palladium solution: 1000- 2000 µg/l
- Ascorbic acid: 5000 µg/l
- Monobasic ammonium phosphate: 5000 µg/l
- Orthophosphoric acid: 1000 µg/l

Preparation of standard curve solutions

In a 100-ml volumetric flask, pipette 25 ml of lead and 10 ml cadmium standards (e and h) and dilute to the mark with water (standard solution A, 1 ml = 25 µg of pb and 1.0 µg of cd). Dilute 10 ml of A to 100 ml with water (standard solution B, 1 ml = 2.5 µg of pb and 0.1 µg Cd). Dilute 10 ml of B to 100 ml with water (standard solution C, 1 ml = 250 ng of pb and 10 ng Cd). Dilute 10 ml of C to 100 ml with water (working standard solution D, 1 ml = 25 ng of pb and 1 ng Cd).

Instrumental conditions

General instrumental conditions are provided in the table below. The recommended settings for the various instrumental parameters vary from model to model, and certain parameters require optimization at the time of use to obtain the best results. Instruments should therefore be adjusted as described in the manufacturer's instructions.

Element	Wave length (nm)	Slit (nm)	Gases	Maximum Ashing Temperature		Atomization Temperature
				Without modifier	With modifier	
Cadmium	228.8	0.5	Argon	300	Argon	1800
Lead	283.3	0.5	Argon	400	Argon	2100

Procedure

Place blank (1% nitric acid), working standard solution (solution D), a suitable modifier solution (if required) and sample solutions in the appropriate locations provided in the furnace auto sampler. Set up the furnace parameters following the instruction provided by the manufacturer to carry out triplicate injections. Clean the graphite tube and inject blank. Program the auto sampler to inject 5, 10, 15, 20 µl of standard (5 µl of modifier and remaining blank solution so as the total volume is 25 µl). Construct the standard curve from

the absorbance either from peak area or height. Inject 10 µl of sample solution and calculate the concentration in the samples as follows:

Injection volume of sample to furnace	:	10 µl
Volume made up	:	50 ml
Instrument reading (ng)	:	R
Weight of sample, g	:	W

Concentration in sample (mg/kg) = (R x 5)/W

Measurement of Arsenic and Antimony by Atomic Absorption Hydride Technique

Arsenic and antimony are determined after preparation of their volatile hydrides which are collected either in the generation vessel itself or, in some designs, in a rubber balloon attached to the vessel. The gases are then expelled with Argon into a hydrogen flame.

Preparation of standard curve solution

Into a series of 100-ml volumetric flasks add from a burette, 0, 1, 2, 3, 4 and 5 ml of standard arsenic or antimony solution [Standards (g) and (d)] and dilute to about 50 ml with distilled water. Add 8 ml 98% sulfuric acid TS and 10 ml hydrochloric acid [1.18 specific gravity]. Shake to dissolve, and when solution is complete, dilute to the mark with distilled water.

Instrumental conditions

Using the atomic absorption spectrophotometer with the appropriate hollow cathode or electrode-less discharge lamp, select the wavelength for either arsenic (193.7 nm) or antimony (217.6 nm).

Procedure

Measure 5.0 ml of the strongest standard into the generation vessel, add 25 ml of water and 2 ml 5 N hydrochloric acid. Stopper the vessel and expel any air as described in the maker's instructions, filling the apparatus with Argon. Isolate the vessel from the atomizer using the by-pass valve. Remove the atomizer and then quickly add 1 pellet (about 0.2 g) of sodium borohydride and replace the stopper. Ensure that all the joints are secure.

When the reaction slows (20 - 30 sec) open the appropriate taps to allow the Argon to drive the generated hydride into the flame. When the hydride has all been expelled as shown by the recorder trace, return the taps to their original position and empty the vessel.

Optimize the instrument settings to give full scale deflection for the strongest standard. Measure the other standards, the sample and the blank solution using the same procedure.

Plot a graph relating peak height on the recorder to concentration of the arsenic or antimony in the standards. Using the net absorbance of the sample, read the concentration of arsenic or antimony in the solution from the graph .

Calculation

Arsenic or antimony in the sample (mg/kg) =
[Concentration of arsenic or antimony (µg/ml) x 50] / [Weight of sample taken (g)]

Determination of Mercury by Cold Vapour Atomic Absorption Technique

Principle

The sample is digested under closed conditions by heating under reflux with sulfuric and nitric acids. The oxidation is completed by addition of potassium permanganate solution. After

successive additions of hydroxylamine hydrochloride solution and stannous chloride solution, the mercury content is measured by cold vapour atomic absorption spectrometry. Alternatively, closed vessel microwave digestion system may be used for the digestion of samples.

Standards

Dilute commercially available mercury standard solution (e.g. 10 µg/ml) following a serial dilution technique (dilution factor in each dilution not to exceed 20) to get 0.02 µg Hg/ml

Apparatus

All the glassware must be cleaned with nitric acid (10% v/v) and washed thoroughly with water before use.

Mineralization apparatus fitted with reflux condenser (see figure 3).

Bubblers, with a ground glass stopper fitted with two tubes to permit entrainment of the mercury vapour and with a calibration mark at the required volume for measurement. The capacity of the bubbler and position of the mark depend on the atomic absorption spectrophotometer used. Clean the bubbler successively with chromic acid mixture (dissolve 4.0 g of potassium dichromate in 300 ml of 3.5 M sulfuric acid and make up to 1 litre with water), tap water and double distilled water before use.

(Alternatively, use the vapour generation accessory and follow operation instructions for its use)

Water vapour absorption apparatus, containing magnesium perchlorate.

Atomic absorption spectrophotometer suitable for the cold vapour determination of mercury in open or closed circuit, with a data station or recorder.

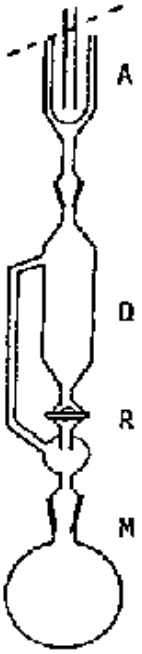


Figure 3. Mineralization Apparatus

Procedure

Digestion of sample

Weigh out, to the nearest 2 mg, approximately 0.5 g sample containing not more than 0.5 µg total mercury. Introduce the sample into the receiver flask (M), and add a few glass beads. Connect the receiver flask to the condensate reservoir (D) and close the stopcock (R).

Introduce into the reservoir 25 ml of nitric acid (sp.gr. 1.40) followed by 10 ml sulfuric acid (sp.gr. 1.84). Mount and turn on the condenser (A). Open the stopcock carefully and allow small portions of the mixture of acids to run into the receiver flask. Interrupt the flow of acids if the reaction becomes too vigorous.

Empty the reservoir into the receiver flask, mix the contents of the latter well by careful shaking and leave the stopcock open.

Heat the receiver flask carefully. As soon as foaming has ceased, close the stopcock (R), continue heating and let the condensate collect in the reservoir.

Discontinue heating when the contents of the receiver flask begin to char. Allow a small portion of the condensate to run into the receiver flask, close the stopcock again and resume heating the receiver flask. Repeat this procedure for as long as the contents display charring when heated.

When charring has ceased, heat and add condensate as soon as white fumes appear. Continue alternately heating and adding condensate for one hour. Finally, heat the contents of the flask to white fumes.

Stop heating and allow to cool to approximately 40°. Open the stopcock and allow all the condensate to run into the receiver flask. Wash the apparatus out from the top of the condenser with 5 - 10 ml of water, collect the washings in the receiver flask and disconnect it from the reservoir.

Treatment of the Solution

Introduce the potassium permanganate solution (50.0 g/l) dropwise into the receiver flask, with agitation, until a pink colouration persists. Note the volume of permanganate solution used. (If this quantity exceeds 10 ml, repeat the procedure "Ashing" as above.)

Heat gently to boiling, then allow to cool.

Pour the contents of the receiver flask into a bubbler, wash the receiver flask with water and add the washings to the contents of the bubbler.

Measure the mercury content (see below) the same day as the treatment of the solution.

Measurement of Mercury Content

Introduce 5 ml of hydroxylamine hydrochloride (100 g/l) into the bubbler and make up to the mark either with double distilled water or with sulfuric acid (3.5 M solution) in the case of standard solutions. Add 5 ml of stannous chloride solution [prepare by dissolving 25.0 g of stannous chloride ($SnCl_2.2H_2O$) in 50 ml hydrochloric acid (sp. gr. 1.18), make up to 250 ml with water and bubble nitrogen through the solution. Store over a few granules of metallic tin], assemble the bubbler, connect it to the water vapour absorption apparatus and to the atomic absorption spectrophotometer. Set the latter in operation.

Mix the contents of the bubbler well by gentle shaking, pass air or nitrogen through, measure and record. Carry out measurements as quickly as possible after the addition of stannous chloride. If an open-circuit system is used, wait 30 sec before passing air or nitrogen.

Standard Curve

Introduce respectively 2-, 5-, 10-, 15- and 25-ml aliquots of the standard mercury solution (0.02 µg Hg/ml) into bubblers and 25 ml sulfuric acid (3.5 M) into a sixth bubbler. Add potassium permanganate solution dropwise, with agitation, to each bubbler until a colouration persists.

Measure the mercury content as described above.

Plot the standard curve with the measured absorption values as ordinates and the corresponding mercury contents in micrograms as abscissae. The working standards contain 0, 0.04, 0.10, 0.20, 0.30 and 0.50 µg of mercury, respectively.

Method of Addition

The method of addition may be used if an open-circuit system is used.

Place one of the working standard solutions in a bubbler and add an aliquot portion of the sample solution obtained after treatment. The quantity of mercury in the bubbler must lie in the range in which the photometer gives a linear response. Measure the mercury content as described above. If necessary, carry out several such determinations, using different working standard solutions.

Blank Determination

Carry out all the operations, from ashing to measurement, except for introduction of the sample. When treating the solution, add a quantity of potassium permanganate solution equal to that used for the experimental sample.

Calculation

Read off from the standard curve the quantities, in µg, of mercury corresponding to the measured absorption values.

Subtract the quantity of mercury found in the blank from that found in the sample.

Net weight of mercury (µg) / sample weight (g) = mg/kg Hg in the sample

Nickel in Polyols

Note: *This method is also applicable for determination of nickel in polydextroses.*

Reagents

Test solution: Dissolve 20.0 g of the sample in a mixture of equal volumes of dilute acetic acid TS and water and dilute to 100 ml with the same mixture of solvents. Add 2.0 ml of a 1% w/v solution of ammonium pyrrolidinedithiocarbamate and 10 ml of methyl isobutyl ketone. Mix and allow the layers to separate and use the methyl isobutyl ketone layer for analysis.

Standard solutions: Prepare three standard solutions in the same manner as the test solution but adding 0.5 ml, 1.0 ml, and 1.5 ml, respectively, of a standard nickel solution containing 10 mg/kg Ni, in addition to the 20.0 g of the sample.

Procedure

Set the instrument to zero using methyl isobutyl ketone prepared as described for the preparation of the test solution but omitting the substance to be examined. Use a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame. The analysis wavelength for all solutions is 232.0 nm.

Nickel Limit Test

Note: *It is recommended to use an appropriate AAS/ICP method in the place of the limit test, if possible, for quantitative determination of nickel.*

Dissolve 10 g of sample in sufficient water to produce 20 ml, add 3 ml bromine TS and 2 ml of a 20% w/v solution of citric acid, mix and add 10 ml of ammonia TS and 1 ml of dimethylglyoxime TS. Mix, dilute to 50 ml with water and allow to stand for 5 min; any colour produced is not more intense than that produced by similarly treating, at the same time, 1 ml of nickel standard solution [10 mg/kg Ni prepared by diluting 1.0 ml of a 0.401% w/v solution of nickel chloride ($NiCl_2 \cdot 6H_2O$ analytical reagent grade) with water to 100.0 ml] diluted to 20 ml with water (0.5 mg/kg Ni).

Nitrogen Determination (Kjeldahl Method)

Caution: *Provide adequate ventilation in the laboratory and do not permit accumulation of exposed mercury.*

Note 1: *The analyst may use commercially available automated Kjeldahl digestion and determination equipment for the determination of Kjeldahl nitrogen.*

Note 2: *All reagents should be nitrogen-free, where available, or otherwise very low in nitrogen content.*

Method I

This method should be used unless otherwise directed in the individual monograph. It is not applicable for certain nitrogen-containing compounds that do not yield their entire nitrogen content upon digestion with sulfuric acid.

Nitrites and Nitrates Absent

Unless otherwise directed, transfer about 1 g of the substance, accurately weighed, to a 500-ml Kjeldahl flask of hard glass, wrapping the sample, if solid or semi-solid, in nitrogen-free filter paper to facilitate the transfer if desired. To the flask add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 ml of sulfuric acid. Gently heat the mixture, keeping the flask inclined at about a 45° angle, and after frothing has ceased, boil briskly until the solution has remained clear green in colour or almost colourless for 30 min. Cool, add 150 ml of water, mix, and cool again. Cautiously pour 100 ml of sodium hydroxide solution (2 in 5) down the inside of the flask so that it forms a layer under the acid solution, then add a few pieces of granulated zinc. Connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser, the delivery tube from which extends well beneath the surface of 50 ml of boric acid solution (1 in 25) contained in a 500-ml flask or bottle. Gently rotate the contents of the Kjeldahl flask to mix, and distil until about two-thirds of the solution has been collected in the receiving flask. To the receiving flask add methyl red/methylene blue TS, and titrate with 0.5 N sulfuric acid. Perform a blank determination substituting 2 g of sucrose for the sample and make the necessary corrections. Each ml of 0.5 N acid is equivalent to 7.003 mg of nitrogen.

Note: *If it is known that the substance to be determined has low nitrogen content, 0.1 N acid may be used in place of the 0.5 N solution, in which case each ml of 0.1 N acid is equivalent to 1.401 mg of nitrogen.*

Nitrites and Nitrates Present

Transfer to a 500-ml Kjeldahl flask of hard glass a quantity of the sample, accurately weighed, representing about 150 mg of nitrogen, add 25 ml of sulfuric acid in which 1 g of salicylic acid has been dissolved, mix, and allow to stand for 30 min, shaking frequently. Add 5 g of powdered sodium thiosulfate, mix, then add 500 mg of powdered cupric sulfate or mercuric oxide, and continue as directed as above, beginning with "Gently heat the mixture...". Prior to the digestion of substances known to have a nitrogen content exceeding 10%, add 500 mg to 1 g of benzoic acid to facilitate decomposition.

Method II (Semi-micro)

Transfer an accurately weighed or measured quantity of the sample equivalent to about 2 or 3 mg of nitrogen, to the digestion flask of a semi-micro Kjeldahl apparatus. Add 1 g of a powdered mixture of potassium sulfate and cupric sulfate (10 to 1), using a fine jet of water to wash down any material adhering to the neck of the flask, then pour 7 ml of sulfuric acid down the inside wall of the flask to rinse it. Add cautiously, down the inside of the flask, 1 ml of 30% hydrogen peroxide, swirling the flask during the addition (**Caution:** *Do not add any peroxide during the digestion.)*

Heat over a free flame or an electric heater until the solution has attained a clear blue colour and the walls of the flask are free from carbonized material. Cautiously add 20 ml of water, cool, then add through a funnel 30 ml of sodium hydroxide solution (2 in 5), and rinse the funnel with 10 ml of water. Connect the flask to a steam distillation apparatus and immediately begin the distillation with steam. Collect the distillate in 15 ml of boric acid solution (1 in 25) to which has been added 3 drops of methyl red/methylene blue TS and enough water to cover the end of the condensing tube. Continue passing the steam until 80 to 100 ml of distillate has been collected, then remove the absorption flask, rinse the end of the condenser tube with a small quantity of water, and titrate with 0.01 N sulfuric acid. Each ml of 0.01 N acid is equivalent to 0.140 mg (140 μg) of nitrogen.

When more than 2 to 3 mg of nitrogen is present in the measured quantity of the substance to be determined, 0.02 or 0.1 N sulfuric acid may be used in the titration if at least 15 ml of titrant is required. If the total dry weight of the material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide added before distillation.

Non-Volatile Residue

Unless otherwise indicated, transfer 100 ml of the sample into a tared 125-ml platinum evaporating dish, previously heated at 105° to constant weight, and evaporate the sample to dryness on a steam bath. Heat the dish at 105° for 30 min or to constant weight, cool in a desiccator, and weigh.

Phosphate Determination as P_2O_5

Method I

Weigh accurately about 200 - 300 mg of sample, dissolve in 25 ml of water and 10 ml of diluted nitric acid TS and boil for 30 min. Filter if necessary, and wash any precipitate, then dissolve the precipitate by the addition of 1 ml diluted nitric acid TS. Adjust the temperature to about 50^{o}, add 75 ml of ammonium molybdate TS, and maintain the temperature at about

50^{o} for 30 min, stirring occasionally. Allow to stand for 16 h or overnight at room temperature. Decant the supernatant, through a filter paper, wash the precipitate once or twice with water by decantation using 30 to 40 ml each time, and pour the washings through the same filter. Transfer the precipitate to the same filter, and wash with potassium nitrate solution (1 in 100) until the filtrate is no longer acid to litmus paper. Transfer the precipitate with filter paper to the original precipitation vessel, add 50.0 ml of 1 N sodium hydroxide, agitate and stir until the precipitate is dissolved, add 3 drops of phenolphthalein TS and titrate the excess alkali with 1 N sulfuric acid. Each ml of 1 N sodium hydroxide consumed is equivalent to 3.088 mg of P_2O_5 .

Method II

Weigh accurately Transfer about 1.5 g of the sample, transfer into accurately weighed, into a 500-ml volumetric flask beaker, add 100 ml of water and 25 ml of nitric acid, and boil for 10 min on a hot plate. Cool, quantitatively transfer into a 500- ml volumetric flask, dilute to volume with water and mix. Pipet 20.0 ml of this solution into a 500- ml Erlenmeyer flask, add 100 ml of water and heat just to boiling. Add with stirring 50 ml of quimociac TS, then cover with a watch glass and boil for 1 min in a well ventilated hood. Cool to room temperature, swirling occasionally while cooling, then filter through a tared crucible (or fritted glass crucible of medium porosity), and wash with five 25-ml portions of water. Dry at about 225° for 30 min, cool and weigh. Each mg of precipitate thus obtained is equivalent to 32.074 µg of P_20_5.

Selenium Limit Test

Reagents

2,3-Diaminonaphthalene Solution: On the day of use, dissolve 100 mg of 2,3-diaminonaphthalene ($C_{10}H_{10}N_2$) and 500 mg of hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$) in sufficient 0.1 N hydrochloric acid to make 100 ml.

Selenium Standard Solution: Transfer 120.0 mg of powdered metallic selenium into a 1,000-ml volumetric flask, and dissolve in 100 ml of dilute nitric acid (1 in 2), warming gently on a steam bath to effect solution. Cool, dilute to volume with water, and mix. Transfer 5.0 ml of this solution into a 200-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 3 µg of selenium (Se).

Alternatively, commercially available selenium standard solution may suitably be diluted to obtain a 3 µg/ml solution.

Note: *Method I referenced in older specifications is deleted – The colourimetric procedure described in Method II may be used, but it is recommended that, whenever possible, the determination of selenium is carried out using atomic absorption methods.*

Method II

Preparation of Standard: Transfer 2.0 ml of the Selenium standard solution into a 150-ml beaker, add 50 ml of 2 N hydrochloric acid, and mix.

Sample Preparation: Transfer into a 150-ml beaker the amount of sample specified in the individual monograph, dissolve in 25 ml of 4 N hydrochloric acid, swirling if necessary to effect solution, heat gently to boiling, and digest on a steam bath for 15 min. Remove from heat, add 25 ml of water, and allow to cool to room temperature.

Procedure

Place the beakers containing the standard preparation and the sample preparation in a fume hood. Cautiously add 5 ml of ammonium hydroxide to each beaker and to a third beaker containing 50 ml of 2 N hydrochloric acid to serve as the blank. Allow the solutions to cool, and then adjust the pH of each solution to 2.0 ± 0.2 with dilute ammonium hydroxide (1 in 2).

Add 200 mg of hydroxylamine hydrochloride to each beaker, swirl gently to dissolve, then without delay add 5 ml of 2.3-diaminonaphthalene solution to each solution, and mix. Cover each beaker with a watch glass, and allow to stand at room temperature for 100 min. Transfer the solutions into separate separators with the aid of about 10 ml of water, extract each solution with 5.0 ml of cyclohexane, shaking each separator vigorously for 2 min, and allow the layers to separate. Discard the aqueous phases, and centrifuge the cyclohexane extracts to remove any traces of water. Determine the absorbance of each extract in a 1-cm cell at the maximum at about 380 nm, with a suitable spectrophotometer, using the blank to set the instrument. The absorbance of the extract from the sample preparation is not greater than that from the standard solution when a 200-mg sample is tested, or not greater than one-half the absorbance of the extract from the standard solution when a 100-mg sample is tested.

Sulfates Limit Test

Unless otherwise specified, place the prescribed quantity of the sample in a Nessler tube, dissolve it in about 30 ml of water, and neutralize with dilute hydrochloric acid TS if the solution is alkaline. Add 1 ml of dilute hydrochloric acid TS and dilute to 50 ml with water. If the use of a sample solution is prescribed, transfer the sample solution into a Nessler tube and dilute to 50 ml with water. Transfer the prescribed volume of 0.01 N sulfuric acid into another Nessler tube to serve as the standard, add 1 ml of dilute hydrochloric acid TS, and dilute to 50 ml with water.

If the solution containing the sample is not clear, filter both solutions under the same conditions. Add 2 ml of barium chloride TS to each solution, mix thoroughly, and allow to stand for 10 min. Compare the turbidity of the two solutions by observing the Nessler tubes from the sides and the tops against a black background. The turbidity of the sample does not exceed that of the standard.

Water-Insoluble Matter

Treat 10 g of sample, accurately weighed, with 100 ml of hot water and filter through a tared filtering crucible. Wash the insoluble residue with hot water, dry at 105° for 2 h, cool and weigh.

Water Determination (Karl Fischer Titrimetric Method)

Note: *Determine the water content by the method below, unless otherwise specified in the individual monograph.*

Principle

The Karl Fischer titrimetric determination of water is based on the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions. In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. Pyridine-free reagents are more commonly used now and are to be preferred in order to avoid the use of pyridine (a hazardous reagent).

The test specimen is titrated with the Karl Fischer Reagent directly. The stoichiometry of the reaction is not exact, and the reproducibility of the determination depends on such factors as the relative concentrations of the Karl Fischer Reagent ingredients, the nature of the inert solvent used to dissolve the test specimen, the apparent pH of the final mixture, and the technique used in the particular determination. Therefore, an empirically standardized technique is used to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system.

The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen; however, other suitable solvents may be used for special or unusual test specimens. Substances that may interfere with the test results are ferric ion, chlorine, and similar oxidizing agents, as well as significant amounts of strong acids or bases, phosgene, or anything that will reduce iodide to iodine, poison the reagent, and show the sample to be bone dry when water may be present (false negative). 8-Hydroxyquinoline may be added to the vessel to eliminate interference from ferric ion. Chlorine interference can be eliminated with SO_2 or unsaturated hydrocarbon. Excess pyridine or other amines may be added to the vessel to eliminate the interference of strong acids. Excess acetic acid or other carboxylic acids can be added to reduce the interference of strong bases. Aldehydes and ketones may react with the solution, showing the sample to be wet while the detector never reaches an endpoint (false positive).

Apparatus

Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and for determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes (about 5 mm^2 in area and about 2.5 cm apart) immersed in the solution to be titrated. At the endpoint of the titration, a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 s to 30 min, depending on the solution being titrated. The time is shortest for substances that dissolve in the Reagent. The longer times are required for solid materials that do not readily go into solution in the Karl Fischer Reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the burette delivering the titrant. A commercially available apparatus generally comprises a closed system consisting of one or two automatic burettes and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant such as phosphorus pentoxide, and the titration vessel may be purged by means of a stream of dry nitrogen or a current of dry air.

Karl-Fischer Reagent

A commercially available, stabilized solution of a Karl Fischer-type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine and/or alcohols other than methanol also may be used. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted Karl Fischer Reagent called for in some monographs should be diluted as directed by the manufacturer. Either methanol, or another suitable solvent such as ethylene glycol monomethyl ether, may be used as the diluent.

Note: *If commercial solution is not available, prepare Karl Fischer Reagent as follows:*

Caution: *Pyridine is hazardous in nature. The analyst shall take proper care and all operations shall be carried out in fume cupboard.*

Add 125 g of iodine to a solution containing 670 ml of methanol and 170 ml of pyridine, and cool. Place 100 ml of pyridine in a 250-ml graduated cylinder, and keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 ml. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One milliliter of this solution, when freshly prepared, is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily in continual use. Protect the solution from light while in use. Store any bulk stock of the solution in a suitably sealed, glass-stoppered container, fully protected from light and under refrigeration.

Test Preparation

Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 10 to 250 mg of water. Where the monograph specifies that the specimen under test is hygroscopic, accurately weigh a sample of the specimen into a suitable container. Use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into the container and shake to dissolve the specimen. Dry the syringe, and use it to remove the solution from the container and transfer it to a titration vessel prepared as directed under Procedure. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured; add this washing to the titration vessel; and immediately titrate. Determine the water content, in milligrams, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, and subtract this value from the water content, in milligrams, obtained in the titration of the specimen under test.

Standardization of the Reagent

Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient Karl Fischer Reagent to give the characteristic color or 100 ± 50 microamperes of direct current at about 200 mV of applied potential. Pure methanol can make the detector overly sensitive, particularly at low mg/kg levels of water, causing it to deflect to dryness and slowly recover with each addition of reagent. This slows down the titration and may allow the system to actually pick up ambient moisture during the resulting long titration. Adding chloroform or a similar non-conducting solvent will retard this sensitivity and can improve the analysis. For determination of trace amounts of water (less than 1%), quickly add 25 µl (25 mg) of pure water, using a 25- or 50-µl syringe, and titrate to the endpoint.

The water equivalence factor F, in milligrams of water per milliliter of reagent, is given by the formula 25/V, in which V is the volume, in milliliters, of the Karl Fischer Reagent consumed in the second titration. For the precise determination of significant amounts of water (more than 1%), quickly add between 25 and 250 mg (25 to 250 µl) of pure water, accurately weighed by difference from a weighing pipet or from a precalibrated syringe or micropipet, the amount of water used being governed by the reagent strength and the burette size, as referred to under Volumetric Apparatus. Titrate to the endpoint.

Calculate the water equivalence factor, F, in milligrams of water per milliliter of reagent by the formula W/V, in which W is the weight, in milligrams, of the water, and V is the volume, in milliliters, of the Karl Fischer Reagent required.

Procedure

Unless otherwise specified, transfer 35 to 40 ml of methanol or other suitable solvent to the titration vessel, and titrate with the Karl Fischer Reagent to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed because it does not enter into the calculations.) Quickly add the test preparation, mix, and again titrate with the Karl Fischer Reagent to the electrometric or visual endpoint.

Calculate the water content of the specimen, in milligrams, by the formula SxF, in which S is the volume, in milliliters, of the Karl Fischer Reagent consumed in the second titration, and F is the water equivalence factor of the Karl Fischer Reagent.

ORGANIC COMPONENTS

Chlorinated Organic Compounds Limit Test

Weigh 0.25 g of the sample to the nearest mg, and dissolve in 10 ml of water. Acidify with nitric acid and filter off the precipitate. Mix the precipitate with 0.5 g of calcium carbonate, dry the mixture and then ignite. Take up the ignition residue in 20 ml of dilute nitric acid TS and filter. Mix the filtrate with 0.5 ml of 0.1 N silver nitrate. The turbidity should not be greater than that obtained by adding 0.5 ml of 0.1 N silver nitrate to a similar volume of dilute nitric acid TS containing the amount of 0.01 N hydrochloric acid prescribed in the individual monograph.

Cyclohexylamine in Cyclamates

Reagents

Methyl orange-boric acid solution: Dissolve 200 mg of methyl orange and 3.5 g of boric acid in 100 ml of water, heating on a steam bath to effect solution. Allow to stand for at least 24 h, and filter before use.

Standard solution: Weigh accurately about 100 mg of cyclohexylamine in a 100-ml volumetric flask, dissolve in 50 ml of water and 0.5 ml of hydrochloric acid TS, dilute to volume with water, and mix. Transfer 5 ml of the solution into a second 100-ml volumetric flask, dilute to volume with water, and mix. Transfer 5 ml of the solution into a third 100-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 2.5 µg of cyclohexylamine.

Test preparation: Prepare the test preparation as directed in the individual monograph.

Procedure

Transfer 10 ml each of the Standard solution and of the Test preparation into two separate 50-ml glass-stoppered centrifuge tubes, and transfer 10 ml of water to a third tube to serve as a blank. To each tube add 3.0 ml of disodium ethylenediaminetetraacetate solution (prepared by dissolving 10 g of disodium ethylenediaminetetraacetate and 3.4 g of sodium hydroxide in 100 ml of water) and 15 ml of a 20:1 mixture of chloroform and n-butanol, shake the tubes for 2 min, and centrifuge. Remove and discard the aqueous layer in each tube, and then transfer 10 ml of the chloroform solution from each tube into separate centrifuge tubes. To each tube add 2 ml of Methyl orange-boric acid solution, shake the tubes for 2 min, and centrifuge. Remove and discard the aqueous layer in each tube, then add to each tube 1 g of anhydrous sodium sulfate, shake well, and allow to settle. Transfer 5 ml of each clear chloroform solution into separate test tubes, add 0.5 ml of 50:1 mixture of methanol and sulfuric acid TS, and mix. Successively determine the absorbance of the solutions in 1 cm cells at 520 nm with a suitable spectrophotometer, using the blank to set the instrument at zero. The absorbance of the solution from the Test preparation does not exceed that from the Standard preparation.

Dicyclohexylamine in Cyclamates

Note: *The procedure uses a packed column GC. In the absence of a packed column GC, capillary GC in the splitless mode, using an equivalent capillary column, may be used. GC conditions need to be established.*

Standard solutions: Weigh accurately about 100 mg of dicyclohexylamine ($C_{12}H_{23}N$, Refractive index (25, D): 1.480-1.488, specific gravity: d (25, 25): 0.905-0.915, boiling point: 254-256º) in a 100-ml volumetric flask, dissolve in chloroform, dilute to volume with chloroform and mix (standard A, 1.0 mg/ml). Transfer 10 ml standard A into a 100-ml volumetric flask, dilute to volume with chloroform and mix (standard B, 100 µg/ml). Into a series of 10 ml volumetric flasks, Transfer 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 ml of standard B solution. Dilute to volume with chloroform, and mix. The working standard solutions contain 10.0, 20.0, 40, 60 and 80.0 µg/ml, respectively.

Procedure

Dissolve 50 g of the sample in 300 ml of water, add 3 ml of sodium hydroxide TS, and extract with 50 ml and 30 ml of chloroform. Combine the extracts, add 2 g of anhydrous potassium carbonate and filter. Wash the container and the residue on the filter paper several times with 5 ml chloroform, combine the washings to the filtrate and concentrate in a rotary evaporator at 30º under vacuum, to about 0.5 ml, quantitatively transfer into a 2 ml volumetric flask, evaporate the solvent in the volumetric flask to about 0.5 ml under a stream of nitrogen, add 1 ml of nitrobenzene standard solution (100 mg in 500 ml chloroform) as an internal standard and make up to the mark with chloroform.

Gas Chromatographic Conditions

Column: Stainless steel, 1.5 m x 3-4 mm i.d., packed with 60-80 mesh diatomaceous earth (gas chromatographic grade) in a solution of methanolic potassium hydroxide. The final potassium hydroxide concentration should be about 3% of the diatomaceous support. Evaporate off the methanol, add a chloroform solution of polyethyleneglycol 6000, and evaporate the chloroform. The content of polyethyleneglycol 6000 should be about 10% of the diatomaceous support.

Carrier gas: Nitrogen or helium, flow rate should be set so that the retention time of nitrobenzene is about 7 min

Injection port, column, and detector temperatures: 225°, 130 -140°, and 250°, respectively.

Standard curve: Prepare a standard curve by mixing 1 ml of each of working standard solution with 1 ml of internal standard solution and analyze by gas chromatography using a flame ionization detector under the conditions described below. Prepare a standard curve by plotting concentration of dicyclohexylamine (in µg per ml), vs. the ratio of the dicyclohexylamine peak area to that of internal standard. Inject the sample solution and calculate the concentration of dicyclohexylamine (in µg per ml,) in the sample solution from the standard curve and calculate the dicyclohexylamine in the sample as follows.

Dicyclohexylamine (mg/kg) = Conc. in sample (µg/ml) x 0.02

1,4-Dioxane and Ethylene Oxide

Determine by headspace gas chromatography using the following procedure:

Stripped sample: Place 3000 g of the sample into a 5000-ml, 4-neck, round-bottom flask equipped with a stirrer, a thermometer, a gas dispersion tube, a dry ice trap, a vacuum outlet, and a heating mantle. At room temperature, evacuate the flask carefully to a pressure of less than 1 mmHg, applying the vacuum slowly while observing for excessive foaming due to entrapped gases. After any foaming has subsided, spurge with nitrogen, allowing the pressure to raise to 10 mmHg. Heat the flask to 60° while increasing the pressure to about 60mmHg. Continue stripping for 4 h, then cool to room temperature. Shut off the vacuum pump, and

bring the flask pressure back to atmospheric while maintaining nitrogen sparging. Remove the sparging tube with the gas still flowing, then turn off the gas flow. Transfer the Stripped sample to a suitable nitrogen-filled container.

Standard Preparations: (*Caution: Ethylene oxide and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.)* Add a suitable quantity of 1,4-dioxane to a known weight of organic-free water in a vial that can be sealed. Determine the amount added by weight difference. Using the special handling described in the following, complete the preparation. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer about 5 ml of the liquid ethylene oxide to a 100-ml beaker chilled in wet ice. Using a gastight gas chromatographic syringe that has been chilled in a refrigerator, transfer a suitable amount of the liquid ethylene oxide into the mixture. Immediately seal the vial, and shake. Determine the amount added by weight difference. By appropriate dilution with Stripped sample, prepare four solutions, covering the range from 1 to 20 mg/kg for the two components added to the matrix (e.g., 5, 10, 15, and 20 mg/kg). Transfer 10 ml of each of these solutions to separate 22-ml pressured headspace vials, seal each with a silicone septum, star spring, and pressure-relief safety aluminium sealing cap, and crimp the cap closed with a cap-sealing tool. Shake for 2 min.

Sample Preparation: Transfer 10 ± 0.01 g of sample to a 22-mL pressure headspace vial, and seal, cap, and crimp as directed for the Standard Preparations.

Apparatus

Gas chromatograph equipped with a balanced pressure automatic headspace sampler and a flame-ionization detector.

Column:	50-m × 0.32-mm fused silica capillary column, or equivalent, bonded with a 5-mm film of 5% phenyl–95% ethylsiloxane, or equivalent.
Column temperature:	Program the column temperature from 70° to 250° at 10°/min
Transfer line temperature:	140°
Detector temperature:	250°
Carrier gas:	Helium
Flow rate:	app. 0.8 ml/min.
Performance:	On the two Calibration plots, no point digresses from its line by more than 10%.

Calibration: Place the vials containing the Standard Preparations in the automated sampler, and start the sequence so that each vial is heated at a temperature of 50° for 30 min before a suitable portion of its headspace is injected into the chromatograph. Set the automatic sampler for a needle withdrawal time of 0.3 min, a pressurization time of 1 min, an injection time of 0.08 min, and a vial pressure of 22 psig with the vial vent off. Obtain the peak areas for ethylene oxide and 1,4-dioxane, which have relative retention times of about 1.0 and 3.1, respectively. Plot the area versus milligram per kilogram on linear graph paper, and draw the best straight line through the points.

Procedure: Place the vial containing the Sample Preparation in the automatic sampler, and chromatograph its headspace as done for the Standard Preparations. Obtain the peak areas of each of the components, and read the concentrations directly from the Calibration plots.

Fumaric and Maleic Acid

Determine by HPLC using the following conditions:

Mobile Phase: Filtered, degassed solution of 0.01 N sulfuric acid in water.

Note: *For all reference standards, do not dry before use, and keep the containers tightly closed and protected from light. Determine the water content of Fumaric Acid Reference Standard titrimetrically before use, and make the necessary correction in preparing the Standard Preparation.*

Standard Preparation: Transfer about 5 mg of Fumaric Acid Reference Standard (USP or equivalent) and about 2mg of Maleic Acid Reference Standard (USP or equivalent), both accurately weighed, into a 1000-ml volumetric flask, dilute to volume with Mobile Phase, and mix.

Sample Preparation: Transfer about 100 mg of sample, accurately weighed, into a 100-ml volumetric flask, dilute to volume with Mobile Phase, and mix.

Resolution Solution: Transfer about 1 g of sample, about 10 mg of Fumaric Acid Reference Standard, and about 4 mg of Maleic Acid Reference Standard, all accurately weighed, into a 1000-ml volumetric flask, dilute to volume with Mobile Phase, and mix.

Apparatus

Liquid chromatograph

Column: 30-cm × 6.5-mm (i.d.) column, or equivalent , packed with a strong cation exchange resin consisting of sulfonated cross-linked styrene–divinylbenzene copolymer in the hydrogen form (Polypore H from Brownlee Lab, or equivalent)
Detection: 210-nm
Column temperature: 37 ± 1°.
Flow rate: App. 0.6 ml/min

Performance

Inject a portion of the Resolution Solution, and obtain the chromatogram. Record the peak responses from the chromatogram. The resolution of the maleic acid and sample peaks is not less than 2.5; the resolution of the fumaric acid and sample peaks is not less than 7.0; and the relative standard deviation of the Sample Solution peak for replicate injections is not more than 2.0%.

Procedure

Separately inject about 20 µl each of the Standard Preparation and the Test Preparation into the chromatograph, record the chromatograms, and measure the peak responses. The relative retention times are approximately 0.6 for maleic acid, approximately 1.0 for malic acid, and approximately 1.5 for fumaric acid. Calculate the quantities, in milligrams, of maleic acid and fumaric acid, in the portion of the sample taken by the formula $100C \times (rU/rS)$, in which C is the concentration, in milligrams per milliliter, of the corresponding Reference Standard in the Standard Preparation, and rU and rS are the responses of the corresponding peaks from the Test Preparation and the Standard Preparation, respectively.

Gum Constituents Identification

Boil a mixture of 200 mg of the sample and 20 ml of 10% sulfuric acid for 3 h. Allow to cool and add excess barium carbonate, mixing with a magnetic stirrer until the solution is pH 7, and filter. Evaporate the filtrate in a rotatory evaporator at 30 - 50° under vacuum until a crystalline (or syrupy) residue is obtained. Dissolve in 10 ml of 40% methanol. This is the hydrolysate. Place 1 to 5 µl spots of hydrolysate on the starting line of two Silica Gel G thin layer plates. On the same plates apply 1 to 10 µg of the reference standards specified in the individual monograph.

Develop one plate in solvent A and one plate in solvent B:

A. A mixture of formic acid, methyl ethyl ketone, tertiary butanol and water (15/30/40/15 by volume) and

B. A mixture of glacial acetic acid/chloroform/water (74/65/11 by volume).

After development spray with a solution of 1.23 g anisidine and 1.66 g phthalic acid in 100 ml ethanol and heat the plates at 100° for 10 min. A greenish yellow colour is produced with hexoses, a red colour with pentoses and a brown colour with uronic acids. Compare sample spots with those for the solutions of the reference standards and identify the constituents specified in the individual monograph.

Norbixin

Determine by HPLC using the following:

Reagents

Dimethylformamide
Acetonitrile
0.1 M NaOH
Methanol
Acetic acid
Norbixin (purity 99 % or higher; prepare according to the procedure in Scotter *et al*. (1994, 1998) as it is not currently available commercially)
Note: *all solvents should be HPLC-grade*

Apparatus:

HPLC system with a suitable pump, injector, and integrator

Column:	Stainless steel; 250 x 4.6 mm
Stationary phase:	Mixed C8/C18 bonded phase, 5 µm or similar
Detector:	UV/visible

HPLC conditions:

Column temperature:	35°
Mobile phase:	Isocratic 65 % Solution A; 35 % Solution B Solution A: acetonitrile; Solution B: 2 % acetic acid (v/v)
Flow rate:	1.0 ml/min
Injection:	10 µl
Detection:	460 nm
Run time:	40 min

Note: *The retention time of norbixin is approximately 10 min*

Procedure:

Standard solution: Weigh accurately about 25 - 50 mg of the norbixin standard and dissolve in 5 ml of 0.1 M NaOH solution. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with methanol.

Sample solution:

Oil-soluble samples: Weigh accurately about 25 - 50 mg of the sample and dissolve in 3 to 5 ml of dimethylformamide. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with acetonitrile.

Water-soluble samples: Weigh accurately about 25 - 50 mg of the sample and dissolve in 5 ml of 0.1 M NaOH solution. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with methanol.

Calculation:

$$\text{Norbixin (\%)} = \frac{A_s \times W_{St} \times P_{St}}{A_{St} \times W_s} \times 100$$

Where:

As is the peak area of the sample solution
ASt is the peak area of the standard solution
PSt is the purity of the standard expressed as a proportion of Norbixin in the norbixin standard (for example, 0.99 if the standard is 99% pure).
WSt is the weight of the standard (mg)
Ws is the weight of the sample (mg)

References:

Scotter, M.J.; Wilson, L.A.; Appleton, G.P. & Castle, L. *Analysis of Annatto (Bixa orellana) food colouring formulations*. 1. J. Agric. Food Chem. 1998, 46, 1031-1038.

Scotter, M.J.; Thorpe, S.A.; Reynolds, S.L.; Wilson, L.A. & Strutt, P.R. *Characterisation of the principal colouring components of Annatto using high performance liquid chromatography with photodiode-array detection*. Food Addit. Contam. 1994, 11, 301-315.

Oxalate Limit Test

Dissolve 0.5 g of sample in 4 ml of water, add 3 ml concentrated hydrochloric acid and then 1 g of granulated zinc. Heat for 1 min in a boiling water bath. Let stand for 2 min at room temperature; decant the supernatant solution into a test tube containing 0.25 ml of a 1% solution of phenylhydrazine hydrochloride. Mix, heat to boiling and cool immediately. Transfer the solution into a glass cylinder with a ground glass stopper and add an equal volume of concentrated hydrochloric acid. Add 0.25 ml of a 5% solution of potassium hexacyanoferrate (III), mix well and let stand for 30 min. The colour of the solution is not more intense than that of a standard solution prepared in the same manner and containing 4.0 ml of a solution of 0.005% oxalic acid in water.

Polyols: Thin Layer Chromatography

Examine by thin layer chromatography (TLC) using silica gel as the coating substance, and using standard and test solutions described in the individual monograph.

Reagents

4-Aminobenzoic acid reagent: Prepare a solution by dissolving 1 g of 4-aminobenzoic acid in a solvent mixture composed of 18 ml acetic acid, 20 ml water and 1 ml phosphoric acid. Prepare this reagent immediately before use.

Sodium periodate reagent: 0.2% w/v sodium periodate in water

Procedure

Apply 2 µl of each of the standard and test solution to the bottom of the TLC plate. Develop the chromatogram over a path of 17 cm using as the mobile phase a mixture of 70 volumes of propanol, 20 volumes of ethyl acetate and 10 volumes of water. Allow the plate to dry in air and spray with a mixture of 2 volumes of 4-aminobenzoic acid reagent with 3 volumes of acetone. Heat at 100^{o} for 15 min. Spray with the sodium periodate reagent. Heat at 100^{o} for 15 min. The principal spot in the chromatogram obtained from the test solution corresponds in position, colour and size to the principal spot obtained from the standard solution.

Pyrrolidone carboxylic acid

Proceed as directed under thin-layer chromatography (see Analytical Techniques) as follows:

Sample: 2 µl of a 0.5 in 100 solution of the sample

Reference: 2 µl of a 0.5 in 100 solution of monosodium L-glutamate containing 2.5 mg of pyrrolidone carboxylic acid

Solvent: A mixture of 2 volumes of n-butanol, 1 volume of glacial acetic acid and 1 volume of water.

Adsorbent: Silica gel

Potassium iodide-starch solution: Stir and heat 0.5 g of starch in about 50 ml of water until it gelatinizes; after cooling add 0.5 g of potassium iodide and water to make up to 100 ml.

Stop the development when the solvent front has advanced about 10 cm from the point of the application dry the plate for 30 min in air.

At the same time, prepare a similar chamber to that used for developing; place a 50-ml beaker containing about 3 g of sodium hypochlorite in the chamber; slowly add 1 ml of hydrochloric acid into the beaker to generate chlorine gas; put on the lid and allow to stand for 30 sec to fill the chamber with chlorine. Place the dried plate in this chamber, put on the lid and allow to stand for 20 min. Take out the plate, keep for 10 min in air and spray with ethanol. After drying, spray with potassium iodide-starch solution and observe the plate under natural light immediately after the standard spot has appeared.

No spot corresponding to pyrrolidone carboxylic acid standard is detected in the sample (sensitivity = 0.2%).

Readily Carbonizable Substances

Procedure

Unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colourless glass resistant to the action of sulfuric acid and contains the specified volume of sulfuric acid TS.

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 min, unless otherwise directed, and compare the colour of the solution with that of the

specified matching fluid in a comparison container which also is of colourless glass and has the same internal and cross-section dimensions. View the fluids transversely against a background of white porcelain or white glass.

When heat is directed in order to effect solution of the substance in the sulfuric acid TS, mix the sample and the acid in a test tube, heat as directed, cool, and transfer the solution to the comparison container for matching.

Matching Fluids

For purposes of comparison, a series of twenty matching fluids, each designated by a letter of the alphabet, is provided, the composition of each being as indicated in the following table. To prepare the matching fluid specified, pipet the prescribed volumes of the colorimetric test solutions (TSC) and water into one of the matching containers, and mix the solutions in the container.

Matching Fluid	Parts of Cobaltous Chloride TSC	Parts of Ferric Chloride TSC	Parts of Cupric Sulfate TSC	Parts of Water
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	8.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

Note: *Solutions A-D are very light brownish-yellow.; solutions E-L are yellow through reddish-yellow; solutions M-O are greenish-yellow; and solutions P-T are light pink.*

Reducing Substances (as Glucose)

Method I (Volumetric)

Transfer about 1 g of the sample, accurately weighed, into a 250-ml Erlenmeyer flask, dissolve in 10 ml of water, and add 25 ml of alkaline cupric citrate TS and cover the flask with a small beaker. Boil gently for exactly 5 min and cool rapidly to room temperature. Add 25 ml of 10% acetic acid solution, 10.0 ml of 0.1 N iodine, 10 ml of dilute hydrochloric acid TS and 3 ml of starch TS, and titrate with 0.1 N sodium thiosulfate to the disappearance of the blue colour. Calculate the content of reducing substances (as D-glucose) by the formula:

% Reducing substances (as D-glucose) = $[(V_1N_1-V_2N_2) \times 2.7] / \text{Sample wt. (g)}$

Where:

V_1 and N_1 are the volume (ml) and normality, respectively, of the iodine solution,
V_2 and N_2 are the volume (ml) and normality, respectively, of the sodium thiosulfate solution, and 2.7 is an empirically determined equivalence factor for D-glucose.

Method II (Gravimetric)

Dissolve 7 g of the sample in 35 ml of water in a 400-ml beaker and mix. Add 25 ml of cupric sulfate TS and 25 ml of alkaline tartrate TS. Cover the beaker with glass, heat the mixture at such a rate that it comes to a boil in approximately 4 min and boils for exactly 2 min. Filter the precipitated cuprous oxide through a tared Gooch crucible previously washed with hot water, ethanol, and ether, and dried at 100° for 30 min. Thoroughly wash the collected cuprous oxide on the filter with hot water, then with 10 ml of ethanol and finally with 10 ml of ether, and dry at 100° for 30 min. The weight of the cuprous oxide does not exceed that prescribed in the individual monograph.

Related Foreign Substances in Flavour Enhancers

Proceed as directed under "Thin-layer chromatography" (see Analytical Techniques) using a sample of the solution described under Method of Assay in the monograph. Use a mixture of 80 volumes of a saturated solution of ammonium sulfate, 18 volumes of a 13.6% w/v solution of sodium acetate and 2 volumes of isopropanol as the developing solvent. Use microcrystalline cellulose as the absorbent. Stop the development when the solvent front has advanced about 10 cm from the point of the application, dry the plate in air, and observe under ultraviolet light (about 254 nm) in a dark place. Only a spot of 5'-guanylic acid or 5'-inosinic acid is detected.

Residual solvents

The solvents listed in the table below can be determined by this method based on headspace gas chromatography. The method may also be used for the determination of isobutyl acetate and methyl acetate. However, information on the approximate retention time for these two solvents is not available.

Solvent	Approximate retention times (min)	Solvent	Approximate retention times (min)
Ethanal	2.81	Ethyl acetate	10.05
Methanol	2.93	Chloroform	10.33
Ethanol	4.09	2-Methyl-1-propanol	11.05
Ethanenitrile	4.55	1-Butanol	12.79
Propanone	4.76	Hexamethyldisiloxane	14.42
2-Propanol	5.23	Propyl acetate	14.97
Ethoxyethane	5.67	4-Methyl-2-pentanone	16.18
2-Methyl-2-propanol	6.21	Pyridine	16.39
Dichloromethane	6.45	3-methyl-2-pentanone	16.90
1-Propanol	7.78	Toluene	18.25
Trimethylsilanol	8.41	Butyl acetate	20.61
2-Butanol	9.61		

Reagents:

Blank: sample with very low solvent content
Internal standard: 3-methyl-2-pentanone
Methanol
Demineralised water

Method I (Determination carried out in water)

Internal standard solution: Add 50.0 ml water to a 50 ml injection vial and seal. Accurately weigh and inject 15 µl 3-methyl-2-pentanone through the septum and reweigh to within 0.01 mg.

Blank solution: Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of water and 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

Samples: Weigh accurately 0.20 g sample into an injection vial. Add 5.0 ml water and add 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

Calibration solution: Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of the water and 1.0 ml of the internal standard solution. Weigh the vial accurately to within 0.01 mg. Inject a known volume of the component of interest through the septum and again reweigh the vial. Heat at 60° for 10 min and shake vigorously for 10 sec.

Method II (Determination carried out in methanol)

Internal standard solution: Add 50.0 ml methanol to a 50 ml injection vial and seal. Accurately weigh and inject 15 µl 3-methyl-2-pentanone through the septum and reweigh to within 0.01 mg.

Blank solution: Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of methanol and 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

Samples: Weigh accurately 0.20 g sample into an injection vial. Add 5.0 ml methanol and add 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

Calibration solution: Solution A: Add 50.0 ml methanol to a 50 ml vial and seal. Accurately weigh, to within 0.01 mg, the vial and inject 50 µl of the component of interest through the septum. Reweigh the vial. Mix well.

Weigh into an injection vial, a known amount of blank (0.20 g), add 4.9 ml of methanol and 1.0 ml internal standard solution. Introduce 0.1 ml of Solution A into the injection vial. Mix well and heat at 60° for 10 min and shake vigorously for 10 sec.

Procedure:

Place the sample, blank and calibration samples in the sample tray of the head-space gas chromatograph – FID system. Analyse using the following analytical conditions.

Column: Fused silica, length 0.8 m, i.d. 0.53mm, coated with DB-wax, film thickness 1 µm

Coupled with: Fused silica, length 30 m, i.d. 0.53 mm, coated with DB-1, film thickness 5 µm

Conditions:

Carrier gas: Helium
Flow rate: 208 kPa, 5 ml/min
Detector: FID
Temperatures
- Injector: 140°
- Oven conditions: 35° for 5 min, then 5°/min to 90°, then 6 min at 90°
- Detector: 300°

Head space sampler
Sample heating temperature: 60°
Sample heating period: 10 min
Syringe temperature: 70°
Transfer temperature: 80°
Sample gas injection: 1.0 ml in split mode

Calculation

$$A \times B \times C / 50 = \text{mg component per injection vial}$$

Where:

A = relative peak area of the component concerned
B = mg internal standard
C = calibration factor

Determination of calibration factors

Method 1:

$$C = D \times 50 / (E \times (F - G))$$

Method 2:

$$C = D / (E \times (F - G) \times 10)$$

Where:

D = mg component weighed
E = mg internal standard
F = relative peak area of component for the calibration solution
G = relative peak area of the same component for the blank solution

Toluenesulfonamides in Saccharines

Determine by gas chromatography (see Analytical Techniques)

Standard and test solutions

Methylene chloride: Use a suitable chromatography grade (or pure solvent obtained by distillation in all-glass apparatus from analytical grade).

Internal standard stock solution: Weigh accurately, about 100 mg of 95% n-tricosane into a 10 ml volumetric flask, dissolve in n-heptane, dilute to volume with the same solvent and mix.

Stock standard preparation: Weigh accurately 20 mg each of reagent grade o-toluene-sulfonamide and p-toluenesulfonamide into a 10 ml volumetric flask, dissolve in methylene chloride, dilute to volume with the same solvent, and mix.

Dilute standard preparations: Pipet into five 10-ml volumetric flasks 0.1, 0.25, 1.0, 2.5 and 5 ml, respectively, of the "Stock standard preparation". Pipet 0.25 ml of the "Internal standard stock solution" into each flask, dilute each to volume with methylene chloride, and mix. These solutions contain 250 µg of n-tricosane, plus respectively, 20, 50, 200, 500 and 1000 µg per ml of each toluenesulfonamide.

Test preparation: Dissolve 2 g of the sample in 8.0 ml of sodium carbonate TS. Mix the solution thoroughly with 10 g of chromatographic siliceous earth (Celite 545 or equivalent). Transfer the mix into a 25 x 250-mm chromatographic tube having a fritted glass disk and a Teflon stopcock at the bottom, and a reservoir at the top. Pack the contents of the tube by tapping the column on a padded surface, and then by tamping firmly from the top. Place 100 ml of methylene chloride in the reservoir, and adjust the stopcock so that 50 ml of eluate is collected in 20-30 min. To the eluate add 25 µl of "Internal standard stock solution". Mix, and then concentrate the solution to a volume of 1 ml in a suitable concentrator tube fitted with a modified Snyder column, using a Kontes tube heater maintained at 90°.

Procedure

Note: *The procedure uses a packed column GC. In the absence of a packed column GC, capillary GC in the splitless mode, using an equivalent capillary column, may be used. GC conditions need to be established.*

Inject 2.5 µl of the "Test preparation" into a suitable gas chromatograph equipped with a flame-ionization detector. The column is of glass, approximately 3 m in length and 2 mm in inside diameter, and it is packed with 3% methyl phenyl silicone in 100 to 120 mesh silanized calcined diatomaceous silica *(***Caution:** *The glass column should extend into the injector for on-column injection and into the detector base to avoid contact with metal)*. The carrier is helium flowing at a rate of 30 ml per min. The injection port, column, and detector are maintained at 225°, 180°, and 250°, respectively. The instrument attenuation setting should be such that 2.5 µl of the "Dilute standard preparation" containing 200 µg per ml of each toluene sulfonamide gives a response of 40-80% of full-scale deflection. Record the chromatogram, note the peaks for o-toluene sulfonamide, p-toluene sulfonamide, and the n-tricosane internal standard, and calculate the areas of each peak by suitable means. The retention times for o-toluene sulfonamide, p-toluene sulfonamide, and n-tricosane are about 5, 6, and 15 min, respectively.

In a similar manner, obtain the chromatograms for 2.5-µl portions of each of the five "Dilute standard preparations", and for each solution determine the areas of the o-toluene sulfonamide, p-toluene sulfonamide, and n-tricosane peaks. From the values thus obtained,

prepare standard curves by plotting concentration of each toluene sulfonamide, in µg per ml, vs. the ratio of the respective toluene sulfonamide peak area to that of n-tricosane. From the standard curve determine the concentration, in µg per ml, of each toluene sulfonamide in the "Test preparation". Divide each value by 2 to convert the result mg/kg of the toluene sulfonamide in 2 g sample taken for analysis.

Note: *If the toluene sulfonamide content of the sample is greater than about 500 mg/kg, the impurity may crystallize out of the methylene chloride concentrate (see "Test preparation"). Although this level of impurity exceeds that permitted by the specifications, the analysis may be completed by diluting the concentrate (usually 1:10 is satisfactory) with methylene chloride containing 250 µg of n-tricosane per ml, and by applying appropriate dilution factors in the calculation. Care must be taken to re-dissolve completely any crystalline toluene sulfonamide to give a homogeneous solution.*

Triphenylphosphine oxide

Determine by HPLC using the following:

Reagents

Hexane
Isopropanol
Tetrahydrofuran (THF)
Triphenylphosphine oxide (TPPO) (purity 99% or higher; ACROS 14043-0250 or equivalent)
Note: *all solvents should be HPLC-grade*

Apparatus:

HPLC system with a suitable pump, injector, and integrator

Column:	Stainless steel; 150 x 4.6 mm
Stationary phase:	Supelcosil LC-Si, 5 µm or similar
Detector:	UV

HPLC conditions:

Column temperature:	20°
Mobile phase:	Isopropanol:hexane (1:24 v/v)
Flow rate:	1.5 ml/min
Injection:	50 µl
Detection:	210 nm
Run time:	10 min

Note: *The retention time of TPPO is approximately 8.1 min*

Procedure:

Standard solution: Weigh accurately about 10 mg of the TPPO standard and dissolve in THF. Transfer quantitatively to a 1000-ml volumetric flask and dilute to volume with THF.

Sample solution: Accurately weigh about 1000 mg of the sample and dissolve in THF. Transfer quantitatively to a 100-ml volumetric flask and dilute to volume with THF.

Calculation:

$$\text{TPPO}\,(\%) = \frac{A_s \times W_{St} \times P_{St} \times 100}{A_{St} \times W_s \times 1000} \times 100$$

Where:

As is the peak area of the sample solution
ASt is the peak area of the standard solution
PSt is the purity of the standard expressed as a proportion of TPPO in the TPPO standard (for example, 0.99 if the standard is 99% pure).
WSt is the weight of the standard (mg)
Ws is the weight of the sample (mg)

MICROBIOLOGICAL ANALYSES

Note: *All methods in this Section reference media and reagents, which are prepared as detailed in the Section entitled "Media, Reagents and Solutions".*

Total (Aerobic) Plate Count

Equipment and materials

1. Work area, level table with ample surface in clean, well-lighted (100 foot-candles at working surface) and well-ventilated room that is reasonably free of dust and drafts. The microbial density, measured in fallout pour plates taken during plating, of air in working area should not exceed 15 colonies /per plate during 15 min exposure.
2. Petri dishes, glass (15 x 100 mm) or plastic (15 x 90 mm).
3. Pipets, 1, 5, and 10 ml, graduated in 0.1 ml units.
4. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps.
5. Water bath, for tempering agar, thermostatically controlled to 45 ± 1°.
6. Incubator, 35 ± 1°.
7. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate.
8. Tally register.
9. Thermometers appropriate range; accuracy checked.
10. Dilution blanks, 90 ± 1 ml Butterfield's phosphate-buffered dilution water

Media and reagents

1. Butterfield's phosphate-buffered dilution water.
2. Plate count agar.

Procedure

Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate, of sample homogenate by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 sec. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 sec if dilution stands more than 3 min before pipeting test portion into petri dish. Add 12-15 ml plate count agar (cooled to 44-46°) to each plate within 15 min of original dilution. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify, invert petri dishes, and incubate promptly for 48 ± 2 h at 35°.

After incubation, count duplicate plates in suitable range (25-250 colonies), using colony counter and tally register; record results per dilution plate counted. Duplicate plates of at least 1 of 3 dilutions should be in 25-250 colony range. When only 1 dilution is in appropriate range, compute average count per g for dilution and report as total plate count per g (see Table 1, Sample No. 1). When 2 dilutions are in appropriate range, determine average count per dilution before averaging 2 dilution counts to obtain total plate count per g (see Table 1, Sample No. 2). If none or only one of duplicate plates of required dilution yields 25-250 colonies, proceed as in "Guidelines", below. Round off counts to two significant figures only at time of conversion to total plate counts. When rounding off numbers, raise second digit to

next higher number only when third digit from left is 5 or greater, and replace dropped digit with zero. If third digit is 4 or less, replace third digit with zero and leave second digit the same.

Guidelines for calculating and reporting total plate counts in uncommon cases

Report all total plate counts computed from duplicate plates containing less than 25 or more than 250 colonies as estimated counts. Use the following as a guide:

- *Plates with fewer than 25 colonies*. When duplicate plates of lowest dilution have fewer than 25 colonies, count actual number on each duplicate of that dilution, average the number of colonies per plate, and multiply by dilution factor to obtain estimated total plate count. Mark total plate count with asterisk to denote that it was estimated from counts outside 25-250 per plate range (see Table 1, Sample No. 3).
- *Plates with more than 250 colonies*. When number of colonies per plate exceeds 250, count colonies in those portions of plate that are representative of colony distribution. Mark calculated total plate count with asterisk to denote that it was estimated from counts outside 25-250 per plate range (see Table 1, Sample No. 4).
- *Spreaders*. Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth such that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreader. Determine average count for each dilution; report arithmetic average of these values as total plate count. (See Table 1, Sample No. 5). When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the total plate count.
- *Duplicate plates, one with 25-250 colonies, the other with more than 250 colonies*. When one plate contains 25-250 colonies and the duplicate contains more than 250 colonies, count both plates and include the plate with more than 250 colonies in computing total plate count (see Table 1, Sample No. 6).
- *Duplicate plates, one plate of each dilution with 25-250 colonies*. When one plate of each dilution contains 25-250 colonies and the duplicate contains more than 250 colonies or fewer than 25 colonies, count all 4 plates and include plates with more than 250 or fewer than 25 colonies in computing the total plate count (see Table 1, Sample No. 7).
- *Duplicate plates, both plates of one dilution with 25-250 colonies and only one duplicate of the other dilution with 25-250 colonies*. When both plates of one dilution contain 25-250 colonies and only one duplicate of the other dilution contains 25-250 colonies, count all 4 plates and include the plate with fewer than 25 or the plate with more than 250 colonies in computing aerobic plate count (see Table 1, Sample No. 8).

- *Plates with no CFU*. When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 25-250 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident (LA).

Table 1. Examples of computation of total plate count (2 plates/dilution poured)

Sample No.	**Colonies counted**			
	1:100	**1:1,000**	**1:10,000**	**Aerobic plate count/g**
1	TNTC	175	16	190,000
	TNTC	208	17	
2	TNTC	224	25	250,000
	TNTC	245	30	
3	18	2	0	1,600*
	14	0	0	
4	TNTC	TNTC	523	5,200,000*
	TNTC	TNTC	487	
5	TNTC	245	35	290,000
	TNTC	230	Spreader	
6	TNTC	245	23	260,000
	TNTC	278	20	
7	TNTC	225	21	270,000
	TNTC	255	40	
8	TNTC	210	18	230,000
	TNTC	240	28	
	TNTC	260	30	270,000
	TNTC	230	28	

*(Asterisk) : estimated count

TNTC : Too numerous to count. Colony count is significantly beyond count range of 250 colonies.

Underlined numbers are used to calculate aerobic plate count.

Spiral Plate Count Method (Alternative Method)

The spiral plate count (SPLC) method for microorganisms uses a mechanical plater to inoculate a rotating agar plate with liquid sample. The sample volume dispensed decreases as

the dispensing stylus moves from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

Equipment and materials

1. Spiral plater
2. Spiral colony counter with special grid for relating deposited sample volumes to specific portions of petri dishes
3. Vacuum trap for disposal of liquids (2-4 liter vacuum bottle to act as vacuum reservoir and vacuum source of 50-60 cm Hg)
4. Disposable micro beakers, 5 ml
5. Petri dishes, plastic or glass, 150 x 15 mm or 100 x 15 mm
6. Plate count agar (standard methods)
7. Calculator (optional), inexpensive electronic hand calculator is recommended
8. Polyethylene bags for storing prepared plates
9. Commercial sodium hypochlorite solution, about 5% NaOCl (bleach)
10. Sterile dilution water
11. Syringe, with Luer tip for obstructions in stylus; capacity not critical
12. Work area, storage space, refrigerator, thermometers, tally, incubator, as described for Conventional Plate Count Method, above.
13. Sodium hypochlorite solution (5.25%). Available commercially.

Preparation of agar plates

Automatic dispenser with sterile delivery system is recommended to prepare agar plates. Agar volume dispensed into plates is reproducible and contamination rate is low compared to hand-pouring of agar in open laboratory. When possible, use laminar air flow hood along with automated dispenser. Pour same quantity of agar into all plates so that same height of agar will be presented to spiral plater stylus tip to maintain contact angle. Agar plates should be level during cooling.

The following method is suggested for pre-pouring agar plates: Use automatic dispenser or pour constant amount (about 15 ml/100 mm plate; 50 ml/150 mm plate) of sterile agar at 60-70° into each petri dish. Let agar solidify on level surface with poured plates stacked no higher than 10 dishes. Place solidified agar plates in polyethylene bags, close with ties or heat-sealer, and store inverted at 0-4.4°. Bring pre-poured plates to room temperature before inoculation.

Preparation of samples.

Samples are prepared as described under Procedures.

Description of spiral plater

Spiral plater inoculates surface of prepared agar plate to permit enumeration of microorganisms in solutions containing between 500 and 500,000 microorganisms per ml. An operator with minimum training can inoculate 50 plates per h. Within the range stated, dilution bottles or pipets and other auxiliary equipment are not required. Required bench space is minimal, and time to check instrument alignment is less than 2 min. Plater deposits decreasing amount of sample in Archimedean spiral on surface of pre-poured agar plate. Volume of sample on any portion of plate is known. After incubation, colonies appear along line of spiral. If colonies on a portion of plate are sufficiently spaced from each other, count

them on special grid which associates a calibrated volume with each area. Estimate number of microorganisms in sample by dividing number of colonies in a defined area by volume contained in same area.

Plating procedure

Check stylus tip angle daily and adjust if necessary. (Use vacuum to hold microscope cover slip against face of stylus tip; if cover slip plane is parallel at about l mm from surface of platform, tip is properly oriented). Liquids are moved through system by vacuum. Clean stylus tip by rinsing for 1 s with sodium hypochlorite solution followed by sterile dilution water for 1 s before sample introduction. This rinse procedure between processing of each sample minimizes cross-contamination. After rinsing, draw sample into tip of Teflon tubing by vacuum applied to 2-way valve. When tubing and syringe are filled with sample, close valve attached to syringe. Place agar plate on platform, place stylus tip on agar surface, and start motor. During inoculation, label petri plate lid. After agar has been inoculated, stylus lifts from agar surface and spiral plater automatically stops. Remove inoculated plate from platform and cover it. Move stylus back to starting position. Vacuum-rinse system with hypochlorite and water, and then introduce new sample. Invert plates and promptly place them in incubator for 48 ± 3 h at 35 ± 1°.

Sterility controls

Check sterility of spiral plater for each series of samples by plating sterile dilution water.

Caution: *Pre-poured plates should not be contaminated by a surface colony or be below room temperature (water can well-up from agar). They should not be excessively dry, as indicated by large wrinkles or glazed appearance. They should not have water droplets on surface of agar or differences greater than 2 mm in agar depth, and they should not be stored at 0-4.4° for longer than l month. Reduced flow rate through tubing indicates obstructions or material in system. To clear obstructions, remove valve from syringe, insert hand-held syringe with Luer fitting containing water, and apply pressure. Use alcohol rinse to remove residual material adhering to walls of system. Dissolve accumulated residue with chromic acid. Rinse well after cleaning.*

Counting grid

Description: Use same counting grid for both 100 and 150 mm petri dishes. A mask is supplied for use with 100 mm dishes. Counting grid is divided into 8 equal wedges; each wedge is divided by four arcs labelled l, 2, 3, and 4 from outside grid edge. Other lines within these arcs are added for ease of counting. A segment is the area between two arc lines within a wedge. Number of areas counted (e.g., 3) means number of segments counted within a wedge. The spiral plater deposits sample on agar plate in the same way each time. The grid relates colonies on spiral plate to the volume in which they were contained. When colonies are counted with grid, sample volume becomes greater as counting starts at outside edge of plate and proceeds toward the center of plate.

Calibration: The volume of sample represented by various parts of the counting grid is shown in the operator's manual that accompanies a spiral plater. Grid area constants have been checked by the manufacturer and are accurate. To verify these values, prepare 11 bacterial concentrations in range of 10^6-10^3 cells/ml by making 1:1 dilutions of bacterial suspension (use a nonspreader). Plate all. Incubate both sets of plates for 48 ± 3 h at 35 ± 1°. Calculate concentrations for each dilution. Count spiral plates over grid surface, using counting rule of 20 (described below), and record number of colonies counted and grid area over which they were counted. Each spiral colony count for a particular grid area, divided by aerobic count/ml

for corresponding spirally plated bacterial concentrations, indicates volume deposited on that particular grid area. Use the following formula:

$$\text{Volume (ml) for grid area} = \frac{Spiral\ colonies\ counted\ in\ area}{Bacterial\ count/ml\ (APC)}$$

Example:

$$\text{Volume (ml)} = \frac{31 + 30\ colonies}{4.1\ x\ 10^{4}\ bacteria/ml}$$

$$= 0.0015 \text{ ml}$$

Examination and reporting of spiral plate counts.

Counting rule of 20. After incubation, center spiral plate over grid by adjusting holding arms on viewer. Choose any wedge and begin counting colonies from outer edge of first segment toward center until 20 colonies have been counted. Complete by counting remaining colonies in segment where 20th colony occurs. Any count irregularities in sample composition are controlled by counting the same segments in the opposite wedge and recording results. Two segments of each wedge were counted on opposite sides of plate with 31 and 30 colonies, respectively. The sample volume contained in the darkened segments is 0.0015 ml. To estimate number of microorganisms, divide count by volume contained in all segments counted.

If 20 CFU are not within the 4 segments of the wedge, count CFU on entire plate. If the number of colonies exceeds 75 in second, third, or fourth segment, which also contains the 20th colony, the estimated number of microorganisms will generally be low because of coincidence error associated with crowding of colonies. In this case, count each circumferentially adjacent segment in all 8 wedges, counting at least 50 colonies, e.g., if the first 2 segments of a wedge contain 19 colonies and the third segment contains the 20th and 76th (or more), count colonies in all circumferentially adjacent first and second segments in all 8 wedges. Calculate contained volume in counted segments of wedges and divide into number of colonies.

When fewer than 20 colonies are counted on the total plate, report results as "less than 500 estimated SPLC per ml." If colony count exceeds 75 in first segment of wedge, report results as "greater than 500,000 estimated SPLC per ml." Do not count spiral plates with irregular distribution of colonies caused by dispensing errors. Report results of such plates as laboratory accident (LA). If spreader covers entire plate, discard plate. If spreader covers half of plate area, count only those colonies that are well distributed in spreader-free areas.

Compute SPLC unless restricted by detection of inhibitory substances in sample, excessive spreader growth, or laboratory accidents. Round off counts as described above. Report counts as SPLC or estimated SPLC per ml.

Coliforms and *E. coli*

Equipment and materials

1. Covered water bath, with circulating system to maintain temperature of 45.5 ± 0.2°. Water level should be above that of medium in immersed tubes
2. Immersion-type thermometer, 1-55°, about 55 cm long, with 0.1° subdivisions, National Bureau of Standards certified, or equivalent
3. Incubator, 35 ± 1°

4. Balance with capacity of >= 2 kg and sensitivity of 0.1 g
5. Blender and blender jar
6. Sterile graduated pipets, 1.0 and 10.0 ml
7. Sterile utensils for sample handling
8. Dilution bottles made of borosilicate glass, with stopper or polyethylene screw caps equipped with Teflon liners. Commercially prepared dilution bottles containing sterile Butterfield's phosphate buffer can also be used.
9. Colony counter
10. pH meter

Media and reagents

1. Brilliant green lactose bile (BGLB) broth, 2%
2. Lauryl tryptose (LST) broth
3. EC broth
4. Levine's eosin-methylene blue (L-EMB) agar
5. Tryptone (tryptophane) broth
6. MR-VP broth
7. Koser's citrate broth
8. Plate count agar (PCA)
9. Butterfield's phosphate-buffered dilution water
10. Kovacs' reagent
11. Voges-Proskauer (VP) reagents
12. Gram stain reagents
13. Methyl red indicator.

Presumptive test for coliform bacteria

Aseptically weigh 10 g sample into sterile, screw-cap jar. Add 90 ml diluent and shake vigorously (50 times through 30 cm arc) to obtain 10^{-1} dilution. Let stand 3-5 min and shake to re-suspend (5 times through 30 cm arc) just before making serial dilutions and inoculations.

Prepare all decimal dilutions with 90 ml sterile dilution water plus 10 ml from previous dilution unless otherwise specified. The dilutions to be prepared depend on the anticipated coliform density. Shake all suspensions 25 times in 30 cm arc for 7 sec. Do not use pipets to deliver <10% of their total volume. Transfer 1 ml portions to 3 LST tubes for each dilution for 3 consecutive dilutions. Hold pipet at angle so that its lower edge rests against tube. Let pipet drain 2-3 sec. Not more than 15 min should elapse from time sample is blended until all dilutions are in appropriate media.

Incubate tubes 48 ± 2 h at 35°. Examine tubes at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate negative tubes for additional 24 h. Examine a second time for gas. Perform a confirmation test on all presumptive positive (gassing) tubes.

Confirmation test for coliforms

Gently agitate each gassing LST tube. Hold the LST tube at angle and insert a loop to avoid transfer of pellicle (if present). Transfer one loopful of suspension to a tube of BGLB broth. Incubate BGLB tubes 48 ± 2 h at 35°. Examine for gas production and record. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for three consecutive dilutions.

Confirmation test for *E. coli*

Gently agitate each gassing LST tube and transfer a loopful of each suspension to tube of EC broth. Incubate EC tubes 48 ± 2 h at 45.5 ± 0.2°. Examine for gas production at 24 ± 2 h; if negative, examine again at 48 ± 2 h. Streak a loopful of suspension from each gassing tube to L-EMB agar. It is essential that 1 portion of plate exhibit well-separated colonies. Incubate 18-24 h at 35°. Examine plates for suspicious *E. coli* colonies, i.e., dark centered with or without metallic sheen. Pick two suspicious colonies from each L-EMB plate and transfer them to PCA agar slants for morphological and biochemical tests. Incubate PCA slants 18-24 h at 35°. If typical colonies are not present, pick 5-10 or more colonies deemed most likely to be *E. coli* , from every plate.

Perform gram stain. Examine all cultures appearing as gram-negative short rods or cocci for the following biochemical activities (the first four tests are collectively termed IMViC):

- *Indole production*. Inoculate tube of tryptone broth and incubate 24 ± 2 h at 35°. Test for indole by adding 0.2-0.3 ml Kovacs' reagent. Appearance of distinct red colour in the upper layer is positive test.
- *Voges-Proskauer-reactive compounds*. Inoculate tube of MR-VP broth and incubate 48 ± 2 h at 35°. Transfer 1 ml to 13 x 100 mm tube. Add 0.6 ml alpha-naphthol solution and 0.2 ml 40% KOH, and shake. Add a few crystal of creatine. Shake and let stand 2 h. Test is positive if eosin pink colour develops.
- *Methyl red-reactive compounds*. Incubate MR-VP tube additional 48 ± 2 h at 35° after Voges-Proskauer test. Add 5 drops methyl red solution to each tube. A distinct red colour is a positive test. Yellow is a negative reaction.
- *Use of citrate*. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate 96 ± 2 h at 35°. Development of distinct turbidity is positive reaction.
- *Production of gas from lactose*. Inoculate tube of LST broth and incubate 48 ± 2 h at 35°. Displacement of medium from inner vial or effervescence after gentle agitation is a positive reaction.

Interpretation. All cultures that (a) ferment lactose with production of gas within 48 h at 35°, (b) appear as Gram-negative non-sporeforming rods or cocci, and (c) give IMViC (the first four tests.) patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*.

Alternatively, MPN determination of *E. coli*, fecal coliforms and coliforms protocols are given below.

Note: *Alternatively, instead of performing the IMViC tests, use commercially prepared biochemical strip tests. Use growth from PCA slants to perform these assays.*

MPN method (Alternative Method)

MPN - Presumptive test for coliforms, fecal coliforms and *E. coli*

Weigh 50 g into sterile high-speed blender jar. Add 450 ml of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile Butterfield's phosphate diluent. The number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25

times in 30 cm arc or vortex mix for 7 s. Do not use pipets to deliver <10% of their total volume. Transfer 1 ml portions to three LST tubes for each dilution for at least three consecutive dilutions. Hold pipet at angle so that its lower edge rests against the tube. Let pipet drain 2-3 s. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

Incubate LST tubes at 35°. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 h. Perform confirmed test on all presumptive positive (gas) tubes.

MPN - Confirmed test for coliforms

From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at 35° and examine for gas production at 48 ± 2 h. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

MPN - Confirmed test for fecal coliforms and *E. coli*

From each gassing LST tube from the Presumptive test, transfer a loopful of suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 45.5° and examine for gas production. If negative, re-incubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis, follow protocol for Completed test for *E. coli* (below).

Note: *Fecal coliform analyses are done at 45.5± 0.2°.*

MPN - Completed test for *E. coli*.

Gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 18-24 h at 35°. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to five suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 h at 35° and use for further testing.

Note: *Identification of any one of the five colonies as E. coli is sufficient to regard that EC tube as positive; hence, not all five isolates may need to be tested.*

Perform Gram stain. All cultures appearing as Gram-negative short rods, should be tested for the IMViC reactions above and also re-inoculated back into LST to confirm gas production.

Salmonella

Equipment and materials

1. Blender and sterile blender jars
2. Sterile, 16 oz (500 ml) wide-mouth, screw-cap jars, sterile 500 ml Erlenmeyer flasks, sterile 250 ml beakers, sterile glass or paper funnels of appropriate size.
3. Sterile, bent glass or plastic spreader rods
4. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g
5. Balance, with weights; 120 g capacity, sensitivity of 5 mg
6. Incubator, 35 ± 2°
7. Refrigerated incubator or laboratory refrigerator, 4 ± 2°
8. Water bath, 49 ± 1°
9. Water bath, circulating, thermostatically-controlled, 43 ± 0.2°
10. Water bath, circulating, thermostatically-controlled,42 ± 0.2°

11. Sterile spoons or other appropriate instruments for transferring food samples
12. Sterile culture dishes, 15 x 100 mm, glass or plastic
13. Sterile pipets, 1 ml, with 0.01 ml graduations; 5 and 10 ml, with 0.1 ml graduations
14. Inoculating needle and inoculating loop (about 3 mm id or 10 5l), nichrome, platinum-iridium, chromel wire, or sterile plastic
15. Sterile test or culture tubes, 16 x 150 mm and 20 x 150 mm; serological tubes, 10 x 75 mm or 13 x 100 mm
16. Test or culture tube racks
17. Vortex mixer
18. Sterile shears, large scissors, scalpel, and forceps
19. Lamp (for observing serological reactions)
20. Fisher or Bunsen burner
21. pH test paper (pH range 6-8) with maximum graduations of 0.4 pH units per color change
22. pH meter

Media and reagents

1. Lactose broth
2. Nonfat dry milk (reconstituted)
3. Selenite cystine (SC) broth
4. Tetrathionate (TT) broth
5. Rappaport-Vassiliadis (RV) medium
6. Xylose lysine desoxycholate (XLD) agar
7. Hektoen enteric (HE) agar
8. Bismuth sulfite (BS) agar
9. Triple sugar iron agar (TSI)
10. Tryptone (tryptophane) broth
11. Trypticase (tryptic) soy broth
12. Trypticase soy broth with ferrous sulfate
13. Lauryl tryptose (LST) broth
14. Trypticase soy-tryptose broth
15. MR-VP broth
16. Simmons citrate agar
17. Urea broth
18. Urea broth (rapid)
19. Malonate broth
20. Lysine iron agar (LIA) (Edwards and Fife)
21. Lysine decarboxylase broth
22. Motility test medium (semisolid)
23. Potassium cyanide (KCN) broth
24. Phenol red carbohydrate broth
25. Purple carbohydrate broth
26. MacConkey agar
27. Nutrient broth
28. Brain heart infusion (BHI) broth
29. Papain solution, 5%
30. Cellulase solution, 1%
31. Tryptose blood agar base
32. Universal preenrichment broth
33. Buffered peptone water
34. Potassium sulfite powder, anhydrous

35. Chlorine solution, 200 mg/kg, containing 0.1% sodium dodecyl sulfate
36. Ethanol, 70%
37. Kovacs' reagent
38. Voges-Proskauer (VP) test reagents
39. Creatine phosphate crystals
40. Potassium hydroxide solution, 40%
41. 1 N Sodium hydroxide solution
42. 1 N Hydrochloric acid
43. Brilliant green dye solution, 1%
44. Bromcresol purple dye solution, 0.2%
45. Methyl red indicator
46. Sterile distilled water
47. Tergitol Anionic 7
48. Triton X-100
49. Physiological saline solution, 0.85% (sterile)
50. Formalinized physiological saline solution
51. *Salmonella* polyvalent somatic (O) antiserum
52. *Salmonella* polyvalent flagellar (H) antiserum
53. *Salmonella* somatic group (O) antisera: A, B, C_1, C_2, C_3, D_1, D_2, E_1, E_2, E_3, E_4, F, G, H, I, Vi, and other groups, as appropriate
54. *Salmonella* Spicer-Edwards flagellar (H) antisera

Procedure

Pre-enrichment

Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth and mix well, essentially preparing a 1:9 sample/broth ratio. Cap jar securely and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°.

Special cases

In some cases, the analysis of samples may be hampered by the viscosity of thickening agents. Additional treatment may be required.

- For gum ghatti, 0.1% of NaCl (final concentration) in lactose broth pre-enrichment medium, adjusted to pH 6.5, is added.
- For the analysis of gelatin, add 5 ml of a 5% papain solution (final concentration of 0.1%) in lactose broth pre-enrichment medium, mix well. Cap jar securely and incubate at 35° for 60 ± 5 min. Mix by swirling and adjust to pH 6.8, if necessary. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°.
- For carob bean gum and guar gum, aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Prepare a 1.0% mannan endo-1,4-betamannosidase (EC 3.2.178) solution (add 1 g mannosidase to 99 ml sterile distilled water). Dispense into 150 ml bottles. Mannosidase solution may be stored at 2-5°C for up to 2 weeks. Add 225 ml sterile lactose broth and 2.25 ml sterile 1% mannosidase solution to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. While vigorously stirring the mannosidase/lactose broth with magnetic stirrer, pour 25 g analytical unit quickly through sterile glass funnel into the mannosidase/lactose broth. Cap jar securely and let stand 60 ± 5 min at room

temperature. Incubate loosely capped container without pH adjustment, for 24 ± 2 h at 35°.

Enrichment

For Carob Bean gum and Guar gum. Transfer 1 ml mixture to 10 ml selenite cystine (SC) broth and another 1 ml mixture to 10 ml TT broth. Vortex. Incubate SC and TT broths 24 ± 2 h at 35°.

For all other samples. Transfer 0.1 ml mixture to 10 ml Rappaport-Vassiliadis (RV) medium and another 1 ml mixture to 10 ml tetrathionate (TT) broth. Vortex.

1. Incubate selective enrichment media as follows:

   High microbial load. Incubate RV medium 24 ± 2 h at 42 ± 0.2° (circulating, thermostatically-controlled, water bath). Incubate TT broth 24 ± 2 h at 43 ± 0.2° (circulating, thermostatically-controlled, water bath).

   Low microbial load (except carob bean gum and guar gum). Incubate RV medium 24 ± 2 h at 42 ± 0.2°C (circulating, thermostatically controlled, water bath). Incubate TT broth 24 ± 2 h at 35 ± 2.0°.

2. Mix (vortex, if tube) and streak 3 mm loopful (10 µl) incubated TT broth on bismuth sulfite (BS) agar (prepare BS plates the day before streaking and store in the dark at room temperature), xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar.
3. Repeat with 3 mm loopful (10 µl) of RV medium (for samples of high and low microbial load foods) and of SC broth (for guar gum).
4. For options of refrigerating incubated sample pre-enrichments and incubated sample selective enrichments (SC and TT broths only) of low moisture foods, see 994.04 in *Official Methods of Analysis,* AOAC International.
5. Incubate plates 24 ± 2 h at 35°.
6. Examine plates for presence of colonies that may be *Salmonella.*

Colony screening

Examine plates as follows:

1. *Typical Salmonella colony morphology*
   - Hektoen enteric (HE) agar: Blue-green to blue colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* species produce yellow colonies with or without black centers.
   - Bismuth sulfite (BS) agar: Typical *Salmonella* colonies may appear brown, grey, or black; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect. Some strains may produce green colonies with little or no darkening of surrounding medium.
   - Xylose lysine desoxycholate (XLD) agar: Pink colonies with or without black centers. Many cultures of *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* species produce yellow colonies with or without black centers.
2. *Atypical Salmonella colony morphology*

In the absence of typical or suspicious *Salmonella* colonies, search for atypical *Salmonella* colonies as follows:

- HE and XLD agars. Atypically a few *Salmonella* cultures produce yellow colonies with or without black centers on HE and XLD agars. In the absence of typical *Salmonella* colonies on HE or XLD agars after 24 ± 2 h incubation, then pick two or more atypical *Salmonella* colonies.
- BS agar. Atypically some strains produce green colonies with little or no darkening of the surrounding medium. If typical or suspicious colonies are not present on BS agar after 24 ± 2 h, then do not pick any colonies but re-incubate an additional 24 ± 2 h. If typical or suspicious colonies are not present after 48 ± 2 h incubation, then pick two or more atypical colonies.

3. *Suggested control cultures*

In addition to the positive control cultures (typical *Salmonella*), three additional *Salmonella* cultures are recommended to assist in the selection of atypical *Salmonella* colony morphology on selective agars. These cultures are a lactose-positive, H_2S-positive *S. diarizonae* (ATCC 12325) and a lactose-negative, H_2S-negative *S. abortus equi* (ATCC 9842); OR a lactose-positive, H_2S-negative *S. diarizonae* (ATCC 29934). These cultures may be obtained from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

Agar slant analysis

1. Select two or more colonies typical or suspected to be *Salmonella* from each selective agar. Inoculate into triple sugar iron (TSI) agar and lysine iron agar (LIA). If BS agar plates have no colonies typical or suspected to be *Salmonella* or no growth whatsoever, incubate them an additional 24 h. Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI agar slant by streaking slant and stabbing butt. Without flaming, inoculate LIA by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm). Store picked selective agar plates at 5-8°.
2. Incubate TSI agar and LIA slants at 35° for 24 ± 2 h. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI agar. In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H_2S in LIA. Some non-*Salmonella* cultures produce a brick-red reaction in LIA slants.
3. If typical colonies are present on the BS agar after 24 ± 2 h incubation, then pick two or more colonies. Irrespective of whether or not BS agar plates are picked at 24 ± 2 h, re-incubate BS agar plates an additional 24 ± 2 h. After 48 ± 2 h incubation, pick two or more typical colonies, if present, from the BS agar plates, only if colonies picked from the BS agar plates incubated for 24 ± 2 h give atypical reactions in triple sugar iron agar (TSI) and lysine iron agar (LIA) that result in culture being discarded as not being *Salmonella* .
4. All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI

should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella*. Test retained, presumed-positive TSI cultures as directed below, to determine if they are *Salmonella* species, including *S. arizonae*. If TSI cultures fail to give typical reactions for *Salmonella* (alkaline slant and acid butt) pick additional suspicious colonies from selective medium plate not giving presumed-positive culture and inoculate TSI and LIA slants as described above.

5. Apply biochemical tests to:

   - Three presumptive TSI agar cultures recovered from set of plates streaked from RV medium, if present, and presumptive TSI agar cultures recovered from plates streaked from tetrathionate broth, if present.
   - If three presumptive-positive TSI cultures are not isolated from one set of agar plates, test other presumptive-positive TSI agar cultures, if isolated, by biochemical and serological tests. Examine a minimum of six TSI cultures for each 25 g analytical unit.

Biochemical and Serological Testing for Salmonella

1. *Mixed cultures*:

   Streak TSI agar cultures that appear to be mixed on MacConkey agar, HE agar, or XLD agar. Incubate plates 24 ± 2 h at 35°. Examine plates for presence of colonies suspected to be *Salmonella*, as follows:

   a. MacConkey agar. Typical colonies appear transparent and colourless, sometimes with dark center. Colonies of *Salmonella* will clear areas of precipitated bile caused by other organisms sometimes present.

   b. Hektoen enteric (HE) agar. See '*Typical Salmonella colony morphology*', above, for procedure.

   c. Xylose lysine desoxycholate (XLD) agar. See '*Typical Salmonella colony morphology*', above, for procedure.

   Transfer at least two colonies suspected to be *Salmonella* to TSI agar and LIA slants as described above, and continue as under '*Agar slant analysis'.*

2. Pure cultures*:*

   a. Urease test (conventional). With sterile needle, inoculate growth from each presumed-positive TSI agar slant culture into tubes of urea broth. Since occasional, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include uninoculated tube of this broth as control. Incubate 24 ± 2 h at 35°.

   b. Optional urease test (rapid). Transfer two 3 mm loopfuls of growth from each presumed-positive TSI agar slant culture into tubes of rapid urea broth. Incubate 2 h in 37 ± 0.5° water bath. Discard all cultures giving positive test. Retain for further study all cultures that give negative test (no change in colour of medium).

3. Serological polyvalent flagellar (H) test*:*

   a. Perform the polyvalent flagellar (H) test at this point, or later, as described below. Inoculate growth from each urease-negative TSI agar slant into 1) brain heart infusion broth and incubate 4-6 h at 35° until visible growth occurs (to test on same day); or 2)

trypticase soy-tryptose broth and incubate 24 ± 2 h at 35° (to test on following day). Add 2.5 ml formalinized physiological saline solution to 5 ml of either broth culture.

b. Select two formalinized broth cultures and test with *Salmonella* polyvalent flagellar (H) antisera. Place 0.5 ml of appropriately diluted *Salmonella* polyvalent flagellar (H) antiserum in 10 x 75 mm or 13 x 100 mm serological test tube. Add 0.5 ml antigen to be tested. Prepare saline control by mixing 0.5 ml formalinized physiological saline solution with 0.5 ml formalinized antigen. Incubate mixtures in 48-50° water bath. Observe at 15 min intervals and read final results in 1 h.

Positive - agglutination in test mixture and no agglutination in control.

Negative - no agglutination in test mixture and no agglutination in control.

Nonspecific - agglutination in both test mixture and control. Test the cultures giving such results with Spicer-Edwards antisera, below.

4. *Spicer-Edwards serological test*:

Use this test as an alternative to the polyvalent flagellar (H) test. It may also be used with cultures giving non-specific agglutination in polyvalent flagellar (H) test. Perform Spicer-Edwards flagellar (H) antisera test as described above. Perform additional biochemical tests (below) on cultures giving positive flagellar test results. If both formalinized broth cultures are negative, perform serological tests on four additional broth cultures (above). If possible, obtain two positive cultures for additional biochemical testing. If all urease-negative TSI cultures from sample give negative serological flagellar (H) test results, perform additional biochemical tests.

5. Testing of urease-negative cultures*:*

a. Lysine decarboxylase broth. If LIA test was satisfactory, it need not be repeated. Use lysine decarboxylase broth for final determination of lysine decarboxylase if culture gives doubtful LIA reaction. Inoculate broth with small amount of growth from TSI agar slant suspicious for *Salmonella*. Replace cap tightly and incubate 48 ± 2 h at 35° but examine at 24 h intervals. *Salmonella* species cause alkaline reaction indicated by purple colour throughout medium. Negative test is indicated by yellow colour throughout medium. If medium appears discoloured (neither purple nor yellow) add a few drops of 0.2% bromcresol purple dye and re-read tube reactions.

b. Phenol red dulcitol broth or purple broth base with 0.5% dulcitol. Inoculate broth with small amount of growth from TSI agar culture. Replace cap loosely and incubate 48 ± 2 h at 35°, but examine after 24 h. Most *Salmonella* species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium. Production of acid should be interpreted as a positive reaction. Negative test is indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) colour throughout medium.

c. Tryptone (or tryptophane) broth. Inoculate broth with small amount of growth from TSI agar culture. Incubate 24 ± 2 h at 35° and proceed as follows:

1) Potassium cyanide (KCN) broth. Transfer 3 mm loopful of 24 h tryptophane broth culture to KCN broth. Heat rim of tube so that good seal is formed when tube is stoppered with wax-coated cork. Incubate 48 ± 2 h at 35° but examine after 24 h. Interpret growth (indicated by turbidity) as positive. Most *Salmonella* species do not grow in this medium, as indicated by lack of turbidity.

2) Malonate broth. Transfer 3 mm loopful of 24 h tryptone broth culture to malonate broth. Since occasional uninoculated tubes of malonate broth turn blue (positive test) on standing, include uninoculated tube of this broth as control. Incubate 48 ± 2 h at 35°, but examine after 24 h. Most *Salmonella* species cultures give negative test (green or unchanged colour) in this broth.

3) Indole test. Transfer 5 ml of 24 h tryptophane broth culture to empty test tube. Add 0.2-0.3 ml Kovacs' reagent. Most *Salmonella* cultures give negative test (lack of deep red colour at surface of broth). Record intermediate, varying shades of orange and pink as ±.

4) Serological flagellar (H) tests for *Salmonella*. If either polyvalent flagellar (H) test (above) or the Spicer-Edwards flagellar (H) test tube test (above) has not already been performed, either test may be performed here.

5) Discard as not *Salmonella* any culture that shows either positive indole test and negative serological flagellar (H) test, or positive KCN test and negative lysine decarboxylase test.

6. *Serological somatic (O) tests for Salmonella*:

Note: *Pre-test all antisera to Salmonella with known cultures*.

a. Polyvalent somatic (O) test.

Using wax pencil, mark off two sections about 1 x 2 cm each on inside of glass or plastic petri dish (15 x 100 mm). Commercially available sectioned slides may be used. Emulsify 3 mm loopful of culture from 24-48 h TSI slant or, preferably, tryptose blood agar base (without blood) with 2 ml 0.85% saline. Add 1 drop of culture suspension to upper portion of each rectangular crayon-marked section. Add 1 drop of saline solution to lower part of one section only. Add 1 drop of *Salmonella* polyvalent somatic (O) antiserum to other section only. With clean sterile transfer loop or needle, mix culture suspension with saline solution for one section and repeat for other section containing antiserum. Tilt mixtures in back-and-forth motion for 1 min and observe against dark background in good illumination. Consider any degree of agglutination a positive reaction. Classify polyvalent somatic (O) test results as follows:

Positive - agglutination in test mixture; no agglutination in saline control.

Negative - no agglutination in test mixture; no agglutination in saline control.

Nonspecific - agglutination in test and in control mixtures. Perform further biochemical and serological tests as described in *Edwards and Ewing's Identification of Enterobacteriaceae* (Ewing, W.H. 1986. Edwards and Ewing's Identification of Enterobacteriacae, 4th ed. Elsevier, New York).

b. Somatic (O) group tests:

Test as in 6a, above, using individual group somatic (O) antisera including Vi, if available, in place of *Salmonella* polyvalent somatic (O) antiserum. For special treatment of cultures giving positive Vi agglutination reaction, refer to sec. 967.28B in *Official Methods of Analysis* (AOAC International). Record cultures that give positive agglutination with individual somatic (O) antiserum as positive for that group. Record cultures that do not react with individual somatic (O) antiserum as negative for that group.

Table 2. Summary of biochemical and serological reactions of *Salmonella*

Test or substrate	Positive	Negative	species reactions [a]
1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3. H_2S (TSI and LIA)	blackening	no blackening	+
4. Urease	purple-red colour	no colour change	-
5. Lysine decarboxylase broth	purple colour	yellow colour	+
6. Phenol red dulcitol broth	yellow colour and/or gas	no gas; no colour change	+[b]
7. KCN broth	growth	no growth	-
8. Malonate broth	blue colour at surface	no colour change	-[c]
9. Indole test	deep red colour at surface	yellow colour at surface	-
10. Polyvalent flagellar test	agglutination	no agglutination	+
11. Polyvalent somatic test	agglutination	no agglutination	+
12. Phenol red lactose broth	yellow colour and/or gas	no gas; no colour change	-[c]
13. Phenol red sucrose broth	yellow colour and/or gas	no gas; no colour change	-
14. Voges-Proskauer test	pink-to-red colour	no colour change	-
15. Methyl red test	diffuse red colour	diffuse yellow colour	+
16. Simmons citrate	growth; blue colour	no growth; no colour change	v

[a]+ is 90% or more positive in 1 or 2 days; - is 90% or more negative in 1 or 2 days; v is variable.

[b]Majority of *S. arizonae* cultures are negative.

[c]Majority of *S. arizonae* cultures are positive.

Classify as *Salmonella* those cultures which exhibit typical *Salmonella* reactions for test Nos. 1-11, shown in Table 2, above. If one TSI culture from 25 g sample is classified as *Salmonella,* further testing of other TSI cultures from the same 25 g sample is unnecessary. Cultures that contain demonstrable *Salmonella* antigens as shown by positive *Salmonella* flagellar (H) test but do not have biochemical characteristics of *Salmonella* should be purified and retested.

Table 3. Criteria for discarding non-*Salmonella* cultures

Test or substrate	Results
1. Urease	positive (purple-red colour)
2. Indole test and Polyvalent flagellar (H) test or Spicer-Edwards flagellar test	positive (violet colour at surface) negative (no agglutination)
3. Lysine decarboxylaseKCN broth	negative (yellow colour) positive (growth)
4. Phenol red lactose broth	positive (yellow colour and/or gas) [a, b]
5. Phenol red sucrose broth	positive (yellow colour and/or gas) [b]
6. KCN broth Voges-Proskauer test Methyl red test	positive (growth) positive (pink-to-red colour) negative (diffuse yellow colour)

[a] Test malonate broth positive cultures further to determine if they are *Salmonella arizonae.*

[b] Do not discard positive broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to determine if they are *Salmonella* species.

Atypical Salmonella Colony Testing

Perform the following additional biochemical tests on cultures that do not give typical *Salmonella* reactions for test Nos. 1-11 in Table 12, above, and that consequently do not classify as *Salmonella* (see Table 23, also above).

a. Phenol red lactose broth or purple lactose broth

1) Inoculate broth with small amount of growth from unclassified 24-48 h TSI agar slant. Incubate 48 ± 2h at 35°, but examine after 24 h. Positive--acid production (yellow colour) and gas production in inner fermentation vial. Consider production of acid only as positive reaction. Most cultures of *Salmonella* give negative test result, indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) colour throughout medium.

2) Discard as not *Salmonella* cultures that give positive lactose tests, except cultures that give acid slants in TSI and positive reactions in LIA or cultures that give positive malonate broth reactions. Perform further tests on these cultures to determine if they are *S. arizonae.*

b. Phenol red sucrose broth or purple sucrose broth

Follow procedure described as directly above. Discard as not *Salmonella*, cultures that give positive sucrose tests, except those that give acid slants in TSI and positive reactions in LIA.

c. MR-VP broth

Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella*. Incubate 48 ± 2 h at 35°.

1) Perform Voges-Proskauer (VP) test at room temperature as follows: Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth an additional 48 h at 35°. Add 0.6 ml α-naphthol and shake well. Add 0.2 ml 40% KOH solution and shake. To intensify and speed reaction, add a few crystals of creatine. Read results after 4 h; development of pink-to-ruby red color throughout medium is positive test. Most cultures

of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red color throughout broth.

2) Perform methyl red test as follows: To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator. Read results immediately. Most *Salmonella* cultures give positive test, indicated by diffuse red color in medium. A distinct yellow color is negative test. Discard, as not *Salmonella*, cultures that give positive KCN and VP tests and negative methyl red test.

d. Simmons citrate agar

Inoculate this agar, using needle containing growth from unclassified TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 h at 35°C. Read results as follows:

Positive - presence of growth, usually accompanied by color change from green to blue. Most cultures of *Salmonella* are citrate-positive.

Negative - no growth or very little growth and no color change.

Alternative Method for Identification of Salmonella

As alternative to conventional biochemical tube system, use any of 5 commercial biochemical systems (API 20E, Enterotube II, *Enterobacteriaceae* II, MICRO-ID, or Vitek GNI) for presumptive generic identification of *Salmonella*. Choose a commercial system based on a demonstration in the analyst's own laboratory of adequate correlation between commercial system and the biochemical tube system outlined in this identification section.

Commercial biochemical kits should not be used as a substitute for serological tests.

Assemble supplies and prepare reagents required for the kit. Inoculate each unit according to Method 978.24 (API 20E, Enterotube II, and Enterobacteriaceae II), sec. 989.12 (MICRO-ID), and Method 991.13 (Vitek GNI) in *Official Methods of Analysis*, incubating for time and temperature specified. Add reagents, observe, and record results. For presumptive identification, classify cultures, according to *Official Methods of Analysis* (AOAC International) as *Salmonella* or not *Salmonella.*

For confirmation of cultures presumptively identified as *Salmonella*, perform the *Salmonella* serological somatic (O) test and the *Salmonella* serological flagellar (H) test or the Spicer-Edwards flagellar (H) test and classify cultures according to the following guidelines:

a. Report as *Salmonella* those cultures classified as presumptive *Salmonella* with commercial biochemical kits when the culture demonstrates positive *Salmonella* somatic (O) test and positive *Salmonella* (H) test.

b. Discard cultures presumptively classified as not *Salmonella* with commercial biochemical kits when cultures conform to AOAC criteria for classifying cultures as not *Salmonella.*

c. For cultures that do not conform to a or b, classify according to additional tests specified above, or additional tests as specified by Ewing, or send to a reference typing laboratory for definitive serotyping and identification.

Treatment of cultures giving negative flagellar (H) test.

If biochemical reactions of certain flagellar (H)-negative culture strongly suggest that it is *Salmonella*, the negative flagellar agglutination may be the result of non-motile organisms or insufficient development of flagellar antigen. Proceed as follows: Inoculate motility test

medium in petri dish, using small amount of growth from TSI slant. Inoculate by stabbing medium once about 10 mm from edge of plate to depth of 2-3 mm. Do not stab to bottom of plate or inoculate any other portion. Incubate 24 h at 35°C. If organisms have migrated 40 mm or more, retest as follows: Transfer 3 mm loopful of growth that migrated farthest to trypticase soy-tryptose broth. Repeat either polyvalent flagellar (H) or Spicer-Edwards serological tests. If cultures are not motile after the first 24 h, incubate an additional 24 h at 35°C; if still not motile, incubate up to 5 days at 25°C. Classify culture as non-motile if above tests are still negative.

Staphylococcus aureus

Direct Plate Count Method

Note: *This method is suitable for the analysis in which more than 100 S. aureus cells/g may be expected. If the analyst suspects that the number of S. aureus cells is below this limit, then the MPN method should be used. If unknown, both procedures can be used.*

Equipment and materials

1. Drying cabinet, laminar air flow or a well–ventilated room that is free of dust and draft with microbial density of the air in working area not exceeding 15 colonies per plate during a 15-minute exposure.
2. Petri dishes , plastic (15 x 90 mm) or glass (15 x 100 mm)
3. Pipets, 1, 5, and 10 ml, graduated in 0.1 ml units
4. Incubator, 35 $\pm$ 1°
5. Sterile bent glass streaking rods or hockey stick, 3-4 mm diameter, 15-20 cm long with an angled spreading surface 45-55 mm long
6. Colony counter, dark-field, Quebec or equivalent, with suitable light source and grid plate.
7. Tally register
8. Sterile test tubes (13 x 100 mm)

Media and reagents

1. Trypticase (tryptone) soy agar (TSA)
2. Baird-Parker medium
3. Sterile coagulase plasma (rabbit) with EDTA (commercially available)
4. Lysostaphin solution
5. Hydrogen peroxide (3%, v/v)
6. Toluidine blue-deoxyribonucleic (DNA) acid agar
7. 0.02 M phosphate-saline buffer containing 1% NaCl
8. Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate
9. Paraffin oil, sterile
10. Phenol Red Carbohydrate Broth

Sample preparation

Under aseptic conditions, prepare serial dilutions of sample by transferring 10 ml of previous dilution to 90 ml of diluent using separate pipets. Avoid sample foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 seconds.

Isolation

For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily adsorbed, place plates upright in incubator for about 1 h. Invert plates and incubate 45-48 h at 35°. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, grey to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasionally from various foods and dairy products, nonlipolytic strains of similar appearance may be encountered, except that surrounding opaque and clear zones are absent. Strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

Enumeration

Count and record colonies. If several types of colonies are observed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. If plates containing >200 colonies have colonies with the typical appearance of *S. aureus* and typical colonies do not appear at higher dilutions, use these plates for the enumeration of *S. aureus*, but do not count nontypical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

Identification of *S. aureus*

a. Coagulase test

Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml TSB containing 10% NaCl and 1% sodium pyruvate broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of TSB suspension. Incubate TSB culture suspension and slants 18-24 h at 35°. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA (B-4, above) to the TSB culture and mix thoroughly. Incubate at 35° and examine periodically over a 6 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further (for this method see: Sperber, W.H. and Tatini, S.R. 1975. Interpretation of the tube coagulase test for identification of *Staphylococcus aureus*. Appl. Microbiol. 29:502-505). Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically. A latex agglutination test (AUREUS TEST™, Trisum Corp., Taipei, Taiwan) may be substituted for the coagulase test if a more rapid procedure is desired.

Ancillary identification tests

a. Catalase test

On a clean glass slide or spot plate, emulsify growth from TSA slant in 3% hydrogen peroxide. Production of gas bubbles shows a positive reaction. Include known positive and negative cultures.

b. Anaerobic utilization of glucose

Inoculate tube of Phenol Red Carbohydrate broth containing glucose (0.5%).

Immediately inoculate each tube heavily with wire loop. Make certain inoculum reaches bottom of tube. Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 35°. Acid is produced anaerobically if indicator changes to yellow throughout tube, indicating presence of *S. aureus*. Run controls simultaneously (positive and negative cultures and medium controls).

c. Anaerobic utilization of mannitol

Repeat b, above, using mannitol as carbohydrate in medium. *S. aureus* is usually positive but some strains are negative. Run controls simultaneously.

d. Lysostaphin sensitivity

Transfer isolated colony from agar plate with inoculating loop to 0.2 ml phosphate-saline buffer, and emulsify. Transfer half of suspended cells to another tube (13 x 100 mm) and mix with 0.1 ml phosphate-saline buffer as control. Add 0.1 ml lysostaphin (dissolved in 100 ml of 0.02 M phosphate-saline buffer containing 1% NaCl for a final concentration of 25 µg lysostaphin/ml) to original tube. Incubate both tubes at 35° for not more than 2 h. If turbidity clears in test mixture, test is considered positive. If clearing has not occurred in 2 h, test is negative. *S. aureus* is generally positive.

e. Thermostable nuclease production

This test is claimed to be as specific as the coagulase test but less subjective, because it involves a colour change from blue to bright pink. It is not a substitute for the coagulase test but rather is a supportive test, particularly for 2+ coagulase reactions. Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. When agar has solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate slides in moist chamber 4 h at 35°. Development of bright pink halo extending at least 1 mm from periphery of well indicates a positive reaction.

Characteristics

Some typical characteristics of two species of staphylococci and the micrococci, which may be helpful in their identification, are listed in Table 1.

Table 1. Typical characteristics of *S. aureus*, *S. epidermidis*, and Micrococci[(a)]			
Characteristic	***S. aureus***	***S. epidermidis***	**Micrococci**
Catalase activity	+	+	+
Coagulase production	+	-	-
Thermonuclease production	+	-	-
Lysostaphin sensitivity	+	+	-
Anaerobic utilization of			
glucose	+	+	-
mannitol	+	-	-
[a] +, Most (90% or more) strains are positive; -, most (90% or more) strains are negative.			

MPN method

Note: *The Most Probable Number (MPN) method is recommended for routine surveillance of products in which small numbers of S. aureus are expected and in foods expected to contain a large population of competing species.*

Equipment and materials - Same as for Direct Plate Count Method.

Media and reagents - Same as for Direct Plate Count Method. Also required: Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate.

Preparation of sample - Same as for Direct Plate Count Method.

Determination of MPN

Inoculate 3 tubes of TSB containing 10% NaCl and 1% sodium pyruvate with 1 ml portions of decimal dilutions of each sample. Highest dilution must give negative endpoint. Incubate tubes 48 ± 2 h at 35°. Using 3 mm loop, transfer 1 loopful from each tube showing growth (turbidity) to plate of Baird-Parker medium with properly dried surface. Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak inoculum to obtain isolated colonies. Incubate plates 48 h at 35°. From each plate showing growth, transfer at least 1 colony suspected to be *S. aureus* to TSB broth (see C of Direct Plate Count Method above). Continue procedure for identification and confirmation of *S. aureus* (see d of Direct Plate Count, above).

Enumeration of Yeasts and Moulds

Equipment and materials

1. Basic equipment (and appropriate techniques) for preparation of sample homogenate
2. Equipment for plating samples
3. Incubator, 25°
4. Arnold steam chest

5. pH meter
6. Water bath, 45 ± 1°

Media and reagents

1. Potato dextrose agar (PDA)
2. Malt extract agar
3. Malt agar (MA)
4. Plate count agar (PCA), standard methods; add 100 mg chloramphenicol/liter when this medium is used for yeast and mould enumeration. This medium is not efficient when "spreader" moulds are present.
5. Antibiotic solutions
6. Dichloran rose bengal chloramphenicol (DRBC) agar
7. Dichloran 18% glycerol (DG18) agar

Procedures

Antibiotics

Antibiotics are added to mycological media to inhibit bacterial growth. Chloramphenicol is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter-sterilized chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

Sample Preparation

Analyze 25-50 g from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Add appropriate amount of 0.1% peptone water to the weighed sample to achieve 10^{-1} dilution, then homogenize in a stomacher for two min. Alternatively, blending for 30-60 sec can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of 10^{-6} should suffice.

Plating and incubation of samples

Spread-plate method: Aseptically pipet 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method: Use sterile cotton-plugged pipet to place 1 ml portions of sample dilutions into prelabelled 15 x 100 mm petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar medium. Mix contents by gently swirling plates clockwise then counter clockwise, taking care to avoid spillage on dish lid. Add agar within 1-2 min after adding dilution. Otherwise, dilution may begin to adhere to dish bottom (especially if sample

is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate, using wide bore pipets. From preparation of first sample dilution to pouring of final plate, no more than 20 min, preferably 10 min, should elapse.

Incubate plates in dark at 25°. Do not stack plates higher than 3 and do not invert. Let plates remain undisturbed until counting.

Counting of plates

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 h. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mould are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (CFU)/g or CFU/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first two digits are an even number (e.g., 445 = 440); round off to digit above if last two digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mould and yeast counts (MYC) as less than 1 times the lowest dilution used.

SPECIFIC METHODS

ENZYME PREPARATIONS

α-Amylase Activity (Bacterial)

Application and Principle

This procedure is used to determine the α-amylase activity, expressed as bacterial amylase units (BAU), of enzyme preparations derived from *Bacillus subtilis* var., *Bacillus licheniformis* var., and *Bacillus stearothermophilus*. It is not applicable to products that contain β-amylase. The assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at 30 ± 0.1°. The degree of hydrolysis is determined by comparing the iodine colour of the hydrolysate with that of a standard.

Apparatus

Use the Reference Colour Standard, the Comparator, and the Comparator Tubes as described under α-Amylase Activity, Fungal, but use either daylight or daylight-type fluorescent lamps as the light source for the Comparator. (Incandescent lamps give slightly lower results.)

Reagents and Solutions

pH 6.6 Buffer: Dissolve 9.1 g of potassium dihydrogen phosphate (KH_2PO_4) in sufficient water to make 1000 ml (Solution A). Dissolve 9.5 g of dibasic sodium phosphate (Na_2HPO_4) in sufficient water to make 1000 ml (Solution B). Add 400 ml of Solution A to 600 ml of Solution B, mix, and adjust the pH to 6.6, if necessary, by the addition of Solution A or Solution B as required.

Dilute Iodine Solution: Prepare as directed under α-Amylase Activity, Fungal.

Special Starch: Use the material described under α-Amylase Activity, Fungal.

Starch Substrate Solution: Disperse 10.0 g (dry-weight basis) of Special Starch in 100 ml of cold water, and slowly pour the mixture into 300 ml of boiling water. Boil and stir for 1 to 2 min, and then cool while continuously stirring. Quantitatively transfer the mixture into a 500 ml volumetric flask with the aid of water, add 10 ml of pH 6.6 Buffer, dilute to volume with water, and mix.

Sample Preparation: Prepare a solution of the sample so that 10 ml of the final dilution will give an endpoint between 15 and 35 min under the conditions of the assay.

Procedure

Pipet 5.0 ml of Dilute Iodine Solution into a series of 13 × 100-mm test tubes, and place them in a water bath maintained at 30 ± 0.1°, allowing 20 tubes for each assay. Pipet 20.0 ml of the Starch Substrate Solution into a 50 ml Erlenmeyer flask, stopper, and equilibrate for 20 min in the water bath at 30°. At zero time, rapidly pipet 10.0 ml of the Sample Preparation into the equilibrated mixture, and continue as directed in the Procedure under α-Amylase Activity, Fungal, beginning with ‘‘. . . mix immediately by swirling, stopper the flask. . . .’’

Calculation

One bacterial amylase unit (BAU) is defined as that quantity of enzyme that will dextrinize starch at the rate of 1 mg/min under the specified test conditions.

Calculate the α-amylase activity of the sample, expressed as BAU, by the formula

$$BAU/g = 40F/T,$$

in which 40 is a factor (400/10) derived from the 400 mg of starch (20 ml of a 2% solution) and the 10 ml aliquot of Sample Preparation used; F is the dilution factor (total dilution volume/sample weight, in grams); and T is the dextrinizing time, in min.

α-Amylase Activity (Fungal)

Application and Principle

This procedure is used to determine the α-amylase activity of enzyme preparations derived from *Aspergillus niger* var.; *Aspergillus oryzae* var.; *Rhizopus oryzae* var.; (and barley malt). The assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at 30 ± 0.1°. The degree of hydrolysis is determined by comparing the iodine colour of the hydrolysate with that of a standard.

Apparatus

Reference Colour Standard: Use a special α-Amylase Color Disk (Orbeco Analytical Systems, 185 Marine Street, Farmingdale, NY 11735, Catalog No. 620-S5 or similar). Alternatively, prepare a colour standard by dissolving 25.0 g of cobaltous chloride ($CoCl_2 \cdot 6H_2O$) and 3.84 g of potassium dichromate in 100 ml of 0.01 N hydrochloric acid. This standard is stable indefinitely when stored in a stoppered bottle or comparator tube.

Comparator: Use either the standard Hellige comparator (Orbeco, Catalog No. 607) or the pocket comparator with prism attachment (Orbeco, Catalog No. 605AHT) or similar. The comparator should be illuminated with a 100-W frosted lamp placed 6 in. from the rear opal glass of the comparator and mounted so that direct rays from the lamp do not shine into the operator's eyes.

Comparator Tubes: Use the precision-bored square tubes with a 13-mm viewing depth that are supplied with the Hellige comparator. Suitable tubes are also available from other apparatus suppliers.

Reagents and Solutions

Buffer Solution (pH 4.8): Dissolve 164 g of anhydrous sodium acetate in about 500 ml of water, add 120 ml of glacial acetic acid, and adjust the pH to 4.8 with glacial acetic acid. Dilute to 1000 ml with water, and mix.

β-Amylase Solution: Dissolve into 5 ml of water a quantity of β-amylase, free from α-amylase activity (Sigma Chemical Co., Catalog No. A7005 or equiv**a**lent), equivalent to 250 mg of β-amylase with 2000° diastatic power.

Special Starch: Use starch designated as ''Starch (Lintner) Soluble'' (Baker Analyzed Reagent, Catalog No. 4010 or equiv**a**lent). Before using new batches, test them in parallel with previous lots known to be satisfactory. Variations of more than ± 3° diastatic power in the averages of a series of parallel tests indicate an unsuitable batch.

Buffered Substrate Solution: Disperse 10.0 g (dry-weight basis) of Special Starch in 100 ml of cold water, and slowly pour the mixture into 300 ml of boiling water. Boil and stir for 1 to 2 min, then cool, and add 25 ml of Buffer Solution, followed by all of the β-Amylase Solution. Quantitatively transfer the mixture into a 500 ml volumetric flask with the aid of water saturated with toluene, dilute to volume with the same solvent, and mix. Store the solution at 30° ± 2° for not less than 18 h but not more than 72 h before use. (This solution is also known as ''buffered limit dextrin substrate.'')

Stock Iodine Solution: Dissolve 5.5 g of iodine and 11.0 g of potassium iodide in about 200 ml of water, dilute to 250 ml with water, and mix. Store in a dark bottle, and make a fresh solution every 30 days.

Dilute Iodine Solution: Dissolve 20 g of potassium iodide in 300 ml of water, and add 2.0 ml of Stock Iodine Solution. Quantitatively transfer the mixture into a 500 ml volumetric flask, dilute to volume with water, and mix. Prepare daily.

Sample Preparation

Prepare a solution of the sample so that 5 ml of the final dilution will give an endpoint between 10 and 30 min under the conditions of the assay.

For barley malt, finely grind 25 g of the sample in a Miag-Seck mill (Buhler-Miag, Inc., P.O. Box 9497, Minneapolis, MN 55440 or similar). Quantitatively transfer the powder into a 1000 ml Erlenmeyer flask, add 500 ml of a 0.5% solution of sodium chloride, and allow the infusion to stand for 2.5 h at 30° ± 0.2°, agitating the contents by gently rotating the flask at 20-min intervals.

Caution: *Do not mix the infusion by inverting the flask. The quantity of the grist left adhering to the inner walls of the flask as a result of agitation must be as small as possible.*

Filter the infusion through a 32-cm fluted filter of Whatman No. 1, or equivalent, paper on a 20-cm funnel, returning the first 50 ml of filtrate to the filter. Collect the filtrate until 3 h have elapsed from the time the sodium chloride solution and the sample were first mixed. Pipet 20.0 ml of the filtered infusion into a 100 ml volumetric flask, dilute to volume with the 0.5% sodium chloride solution, and mix.

Procedure

Pipet 5.0 ml of Dilute Iodine Solution into a series of 13 × 100-mm test tubes, and place them in a water bath maintained at 30° ± 0.1°, allowing 20 tubes for each assay.

Pipet 20.0ml of the Buffered Substrate Solution, previously heated in the water bath for 20 min, into a 50 ml Erlenmeyer flask, and add 5.0 ml of 0.5% sodium chloride solution, also previously heated in the water bath for 20 min. Place the flask in the water bath.

At zero time, rapidly pipet 5.0 ml of the Sample Preparation into the equilibrated substrate, mix immediately by swirling, stopper the flask, and place it back in the water bath. After 10 min, transfer 1.0 ml of the reaction mixture from the 50 ml flask into one of the test tubes containing the Dilute Iodine Solution, shake the tube, then pour its contents into a Comparator Tube, and immediately compare with the Reference Colour Standard in the Comparator, using a tube of water behind the colour disk.

Note: *Be certain that the pipet tip does not touch the iodine solution as carry-back of iodine to the hydrolyzing mixture will interfere with enzyme action and will affect the results of the determination.*

In the same manner, repeat the transfer and comparison procedure at accurately timed intervals until the α-amylase colour is reached, at which time record the elapsed time. In cases where two comparisons 30 s apart show that one is darker and the other lighter than the Reference Colour Standard, record the endpoint to the nearest quarter min. Shake out the 13-mm Comparator Tube between successive readings. Minimize slight differences in colour discrimination between operators by using a prism attachment and by maintaining a 15- to 25-cm. distance between the Comparator and the operator’s eye.

Calculation

One α-amylase dextrinizing unit (DU) is defined as the quantity of α-amylase that will dextrinize soluble starch in the presence of an excess of β-amylase at the rate of 1 g/h at 30°.

Calculate the α-amylase dextrinizing units in the sample as follows:

$$DU\ (solution) = 24/(W \times T),\ and$$

$$DU\ (dry\ basis) = DU\ (solution) \times 100/(100 - M),$$

in which W is the weight, in grams, of the enzyme sample added to the incubation mixture in the 5 ml aliquot of the Sample Preparation used; T is the elapsed dextrinizing time, in minutes; 24 is the product of the weight of the starch substrate (0.4 g) and 60 min; and M is the percent moisture in the sample, determined by suitable means.

Antibacterial Activity

Scope

This procedure is designed for the determination of antibacterial activity in enzyme preparation derived from microbial sources.

Principle

The assay is based on the measurement of inhibition of bacterial growth under specific circumstances.

Culture Plates

Six organisms are tested: *Staphylococcus aureus* (ATCC 6538); *Escherichia coli* (ATCC 11229); *Bacillus cereus* (ATCC 2); *Bacillus circulans* (ATCC 4516); *Streptococcus pyrogenes* (ATCC 12344): and *Serratia marcescens* (ATCC 14041).

Make a test plate of each organism by preparing a 1:10 dilution of a 24 h Trypticase Soya Broth culture in Trypticase Agar (TSA) (for *Streptococcus pyrogenes* a 1:20 dilution).

Pour 15 ml of plain TSA into a Petri dish and allow the medium to harden. Overlay with 10 ml of seeded TSA and allow to solidify. Place a paper disk prepared according to Disk Preparation of the tested enzyme on each of the six inoculated plates.

Disk Preparation

Make a 10% solution of the enzyme by adding 1 g of enzyme to 9 ml of sterile, distilled water.

Mix thoroughly with a Vortex mixer to obtain a homogeneous suspension. Autoclave suitable paper disks (for instance, S & S Analytical Filter Papers No. 740-E, 12.7 mm in diameter), then saturate them with the enzyme by application of 0.1 ml (about 3 drops) of a 10% solution of the enzyme to the disk surface. Prepare six disks (one for each of the six organisms) for each enzyme: place one disk on the surface of the six inoculated agar plates.

Incubation

Keep the six plates in the refrigerator overnight to obtain proper diffusion. Incubate the plates at 37° for 24 h. Examine the plates for any inhibition zones that may have been caused by the enzyme preparation.

Interpretation

A visually clear zone around a disk (total diameter: 16 mm) indicates the presence of antibacterial components in the enzyme preparation. If an enzyme preparation shows obvious antibacterial activity against three (or more) organisms, it is concluded that antimicrobial agents are present.

Catalase Activity

Scope

This procedure is designed for the determination of catalase activity, expressed as Baker Units.

Principle

The assay is an exhaustion method based on the breakdown of hydrogen peroxide by catalase, and the simultaneous breakdown of the catalase by the peroxide, under controlled conditions.

Reagents and Solutions

0.250 N Sodium thiosulfate: Dissolve 62.5 g of sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$ in 750 ml of recently boiled and cooled water, add 3.0 ml of 0.2 N sodium hydroxide as a stabilizer, dilute to 1,000 ml with water, and mix. Standardize as directed for 0.1 N Sodium thiosulfate (Volumetric Solutions), and adjust to exactly 0.250 N if necessary.

Peroxide substrate solution: Dissolve 25.0 g of anhydrous dibasic sodium phosphate (Na_2HPO_4), or 70.8 g of $Na_2HPO_4 \cdot 12H_2O$, in about 1,500 ml of water, and adjust to pH 7.0 ± 0.1 with 85% phosphoric acid. Cautiously add 100 ml of 30% hydrogen peroxide, dilute to 2,000 ml, in a graduate, and mix. Store in a clean amber bottle, loosely stoppered. The solution is stable for more than one week if kept at 5° in a full container.

Note: *With freshly prepared substrate, the blank will require about 16 ml of 0.250 N sodium thiosulfate. If the blank requires less than 14 ml, the substrate solution is unsuitable and should be prepared fresh again. It is essential that the sample titration is between 50% and 80% of that required for the blank.*

Procedure

Pipet an aliquot of not more than 1.0 ml of the sample, previously diluted to contain approximately 3.5 Baker Units of catalase, into a 200-ml beaker. Rapidly add 100 ml of Peroxide Substrate Solution, previously adjusted to 25°, and stir immediately for 5 to 10 sec. Cover the beaker, and incubate at 25 ± 1° until the reaction is completed. Stir vigorously for 5 sec and then pipet 4.0 ml from the beaker into a 50-ml Erlenmeyer flask. Add 5 ml of 2 N sulfuric acid to the flask, mix, then add 5.0 ml of 40% potassium iodide, freshly prepared, and 1 drop of 1% ammonium molybdate and mix. While continuing to mix, titrate rapidly to a colourless endpoint with 0.250 N Sodium thiosulfate, recording the required volume, in ml, as S. Perform a blank determination with 4.0 ml of Peroxide Substrate Solution, and record the required volume, in ml, as B.

Note: *When preparations derived from beef liver are tested, the reaction is complete within 30 min. Preparations derived from Aspergillus and other sources may require up to 1 h. In assaying an enzyme of unknown origin, a titration should be run after 30 min and then at 10 min intervals thereafter. The reaction is complete when two consecutive titrations are the same.*

Calculation

One Baker Unit is that amount of catalase that will decompose 266 mg of hydrogen peroxide under the conditions of the assay. Calculate the activity of the sample by the formula:

$$\text{Baker Units per g or ml} = 0.4\,(B - S) \times (1/C)$$

in which C is the ml of aliquot of original enzyme preparation added to each 100 ml of Peroxide Substrate Solution, or, when 1 ml of diluted enzyme is used, C is the dilution factor.

Cellulase Activity

Application and Principle

This assay is based on the enzymatic hydrolysis of the interior β-1,4-glucosidic bonds of a defined carboxymethylcellulose substrate at pH 4.5 and at 40°. The corresponding reduction in substrate viscosity is determined with a calibrated viscometer.

Apparatus

Calibrated Viscometer: Use a size 100 Calibrated Cannon-Fenske Type Viscometer, or its equivalent (Scientific Products, Catalog No. P2885-100).

Constant-Temperature Glass Water Bath: (40 ± 0.1°) Use a constant-temperature glass water bath, or its equivalent

Stopwatches: Use two stopwatches, Stopwatch No. 1, calibrated In 1/10 min for determining the reaction time (Tr), and Stopwatch No. 2, calibrated in 1/5 s for determining the efflux time (Tt).

Waring Blender: Use a two-speed Waring blender, or its equivalent (Scientific Products, Catalog No. 58350-1).

Reagents and Solutions

Acetic Acid Solution (2 N): While agitating a 1 l beaker containing 800 ml of water, carefully add 116 ml of glacial acetic acid. Cool to room temperature. Quantitatively transfer the solution to a 1 l volumetric flask, and dilute to volume with water.

Sodium Acetate Solution (2 N): Dissolve 272.16 g of sodium acetate trihydrate in approximately 800 ml of water contained in a 1 liter beaker. Quantitatively transfer to a 1 liter volumetric flask, and dilute to volume with water.

Acetic Acid Solution (0.4 N): Transfer 200 ml of Acetic Acid Solution (2 N) into a 1 liter volumetric flask, and dilute to volume with water.

*Sodium Acetate Solution (0.4 N)***:** Transfer 200 ml of Sodium Acetate Solution (2 N) into a 1 liter volumetric flask, and dilute to volume with water.

Acetate Buffer (pH 4.5): Using a standardized pH meter, add Sodium Acetate Solution (0.4 N) with continuous agitation to 400 ml of Acetic Acid Solution (0.4 N) in a suitable flask until the pH is 4.5 ± 0.05.

Sodium Carboxymethylcellulose: Use sodium carboxymethylcellulose (Hercules, Inc., CMC Type 7HF or equiv**a**lent).

Sodium Carboxymethylcellulose Substrate (0.2% w/v): Transfer 200 ml of water into the bowl of the Waring blender. With the blender on low speed, slowly disperse 1.0 g (moisture-free basis) of the Sodium Carboxymethylcellulose into the bowl, being careful not to splash out any of the liquid. Using a rubber policeman to assist, wash down the sides of the glass

bowl with water. Place the top on the bowl and blend at high speed for 1 min. Quantitatively transfer to a 500-ml volumetric flask, and dilute to volume with water. Filter the substrate through gauze before use.

Sample Preparation

Prepare an enzyme solution so that 1 ml of the final dilution will produce a relative fluidity change between 0.18 and 0.22 in 5 min under the conditions of the assay. Weigh the enzyme, and quantitatively transfer it to a glass mortar. Triturate with water and quantitatively transfer the mixture to an appropriate volumetric flask. Dilute to volume with water, and filter the enzyme solution through Whatman No. 1 filter paper before use.

Procedure

Place the Calibrated Viscometer in the 40 ± 0.1° water bath in an exactly vertical position. Use only a scrupulously clean viscometer. (To clean the viscometer, draw a large volume of detergent solution followed by water through the viscometer by using an aspirator with a rubber tube connected to the narrow arm of the viscometer.) Pipet 20 ml of filtered Sodium Carboxymethylcellulose Substrate and 4 ml of Acetate Buffer into a 50-ml Erlenmeyer flask. Allow at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks, and equilibrate them in the water bath for 15 min. At zero time, pipet 1 ml of the enzyme solution into the equilibrated substrate. Start stopwatch no. 1, and mix the solution thoroughly. Immediately pipet 10 ml of the reaction mixture into the wide arm of the viscometer. After approximately 2 min, apply suction with a rubber tube connected to the narrow arm of the viscometer, drawing the reaction mixture above the upper mark into the driving fluid head. Measure the efflux time by allowing the reaction mixture to freely flow down past the upper mark. As the meniscus of the reaction mixture falls past the upper mark, start stopwatch no. 2. At the same time, record the reaction time, in minutes, from stopwatch no. 1 (Tr). As the meniscus of the reaction mixture falls past the lower mark, record the time, in seconds, from stopwatch no. 2 (Tt).

Repeat the final step until a total of four determinations are obtained over a reaction time (Tr) of not more than 15 min.

Prepare a substrate blank by pipetting 1 ml of water into 24 ml of buffered substrate. Pipet 10 ml of the reaction mixture into the wide arm of the viscometer. Determine the time (Ts) in seconds required for the meniscus to fall between the two marks. Use an average of five determinations for (Ts).

Prepare a water blank by pipetting 10 ml of equilibrated water into the wide arm of the viscometer. Determine the time (Tw) in seconds required for the meniscus to fall between the two marks. Use an average of five determinations for (Tw).

Calculations

One Cellulase Unit (CU) is defined as the amount of activity that will produce a relative fluidity change of 1 in 5 min in a defined carboxymethylcellulose substrate under the conditions of the assay.

Calculate the relative fluidities (Fr) and the (Tn) values for each of the four efflux times (*T*t) and reaction times (*T*r) as follows:

$$Fr = (Ts - Tw)/(Tt - Tw),$$

$$Tn = 1/2(Tt/60\ s/min) + Tr = (Tt/120) + Tr,$$

in which

Fr is the relative fluidity for each reaction time;
Ts is the average efflux time, in seconds, for the substrate blank;
Tw is the average efflux time, in seconds, for the water blank;
Tt is the efflux time, in seconds, of reaction mixture;
Tr is the elapsed time, in minutes, from zero time, that is, the time from addition of the enzyme solution to the buffered substrate until the beginning of the measurement of efflux time (Tt);
Tn is the reaction time, in minutes (*T*r), plus one-half of the efflux time (*T*t), converted to minutes.

Plot the four relative fluidities (Fr) as the ordinate against the four reaction times (Tn) as the abscissa. A straight line should be obtained. The slope of this line corresponds to the relative fluidity change per minute and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a single relative fluidity value. From the graph, determine the Fr values at 10 and 5 min. They should have a difference in fluidity of not more than 0.22 or less than 0.18. Calculate the activity of the enzyme unknown as follows:

$$CU/g = [1000(Fr10 - Fr5)]/W,$$

in which

Fr5 is the relative fluidity at 5 min of reaction time;
Fr10 is the relative fluidity at 10 min of reaction time;
1000 is the milligrams per gram;
W is the weight, in mg of enzyme added to the reaction mixture in a 1-ml aliquot of enzyme solution.

Ethylenimine Limit Test

Scope

This procedure is designed to detect the presence of ethylenimine in immobilized enzyme preparations containing poly(ethylenimine).

Principle

The principle of the method is to react any free ethylenimine which may be present in a sample of immobilized enzyme preparation with an aqueous solution of 1,2-naphthoquinone-4-sulfonate (Folin's reagent) to produce 4-(1-aziridinyl)-1,2-naphthoquinone. This reaction product is extracted into chloroform and the extract analyzed by high performance liquid chromatography (HPLC).

Apparatus

- High performance liquid chromatograph equipped with an ultraviolet detector (254 nm), injection valve and Lichrosorb DIOL column, 5 nm, 4.6-mm i.d. x 25-cm (or equivalent)
- Glass syringe 10 µl
- Separatory funnel, 100 ml
- Pipettes of convenient volumes for the preparation of standard solutions.

Reagents and Solutions

Chloroform with 1% ethanol as a stabilizer, UV grade, distilled in glass
Hexane, UV grade, distilled in glass
2-propanol, UV grade, distilled in glass
Methyl alcohol, UV grade, distilled in glass
Acetone, UV grade, distilled in glass
1,2-naphthoquinone-4-sulfonic acid, sodium salt
0.1 N sodium hydroxide (NaOH)
0.1 M Potassium dihydrogen phosphate (KH_2PO_4)

Buffer Solution: pH 7.7; mix 200 ml of 0.1 M KH_2PO_4 with 93.4 ml of 0.1 N NaOH.

Folin's Reagent: Dissolve 0.40 g of 1,2-naphtoquinone-4-sulfonic acid sodium salt in 100 ml of buffer solution. Dilute to 500 ml with distilled water in a volumetric flask. Wrap the flask in aluminium foil and store in the refrigerator. Discard the reagent after five days.

4-(1-Aziridinyl)-1,2-naphthoquinone

A standard sample of known purity is required. If a commercial source for this standard is not readily available, the substance may by synthesized by the following procedure:

Wrap a separatory funnel with aluminium foil and add 2 g of the sodium salt of 1,2-naphthoquinone-4-sulfonic acid dissolved in 250 ml of distilled water.

Add 25 ml of 0.5 M trisodium phosphate, shake and check that the pH is between 10.5 and 11.5. Add 0.3 ml ethylenimine and shake intermittently for 10 min.

Caution: *Ethylenimine has been identified as a carcinogen. Appropriate precautions must be taken in handling the compound to avoid personnel exposure and area contamination.*

Extract the 4-(aziridinyl)-1,2-naphthoquinone formed with six 200-ml portions of chloroform.

Place the combined extracts in a 2-liter beaker wrapped in aluminium foil in which three holes have been made.

Evaporate the chloroform at room temperature with a nitrogen purge. Transfer the dry residue to a 50-ml beaker wrapped in aluminium foil.

Add 35 ml of methyl alcohol and 1 ml of chloroform to the residue and stir briefly. Not all of the residue will dissolve.

Place the beaker in an ice-water bath for 10 min and then filter the precipitate through Whatman 42 filter paper.

Rinse the precipitate in the filter with 4 ml of chilled methyl alcohol and discard the filtrates.

Dry the precipitate with a nitrogen purge, transfer it to a brown glass bottle and purge again. Dry the compound overnight in a desiccator containing Drierite. The melting point of the compound is 173-175°. The compound is to be used for making standard solutions for calibration purposes. The compound should be stored in a freezer until standard solutions are to be prepared.

0.5 g/l Standard Solution: Accurately weigh about 125 mg of 4-(1-aziridinyl)-1,2-naphthoquinone into a 250 ml volumetric flask [low actinic glass] and add chloroform to the mark.

0.1 mg/l Standard Solution: By appropriate dilution(s) of the 0.5 g/l Standard Solution, prepare a standard solution which contains 0.1 mg/L (0.1 ng/µl).

Analysis

Accurately weigh a sample of immobilized enzyme preparation containing about 10 g of dry matter into an aluminium foil-covered beaker. Add 50 ml of Folin's Reagent and agitate the mixture for several minutes. Decant the Folin's Reagent into a separatory funnel and extract with 2 ml of chloroform. Analyze a 20 µl portion of the chloroform extract by the following chromatographic conditions:

Column: Lichrosorb DIOL 5 nm (or equivalent)

Mobile phase: hexane:chloroform (with 1% ethanol) : isopropanol = 59.5 : 40.0 : 0.5 (v/v)

Flow rate: 2 ml/min.

Inject a 20 µl portion of the 0.1 mg/L Standard Solution. The sample response is not greater than that of the 0.1 mg/L Standard Solution. (Another sample containing a standard addition of 4-(1-aziridinyl)-1,2-naphthoquinone to immobilized enzyme preparation should be analyzed to verify that the chromatographic response does not contain interfering substances.)

β-Galactosidase (Lactase) Activity

Application and Principle

This procedure is used to determine β-Galactosidase activity of enzyme preparations derived from *Aspergillus oryzae* var. The assay is based on a 15-min hydrolysis of an *o*-nitrophenyl-b-D-galactopyranoside substrate at 37° and pH 4.5.

Reagents and Solutions

2.0 N Acetic Acid: Dilute 57.5 ml of glacial acetic acid to 500 ml with water. Mix well, and store in a refrigerator.

4.0 N Sodium Hydroxide: Dissolve 40.0 g of sodium hydroxide in sufficient water to make 250 ml.

Acetate Buffer: Combine 50 ml of 2.0 N Acetic Acid and 11.3 ml of 4.0 N Sodium Hydroxide in a 1000-ml volumetric flask, and dilute to volume with water. Verify that the pH is 4.50 6 0.05, using a pH meter, and adjust, if necessary, with 2.0 N Acetic Acid or 4.0 N Sodium Hydroxide.

2.0 mM o-Nitrophenol Stock: Transfer 139.0 mg of *o*-nitrophenol a 500-ml volumetric flask, dissolve in 10 ml of USP alcohol (95% ethanol) by swirling, and dilute to volume with 1% sodium carbonate.

o-Nitrophenol Standards

0.10 mM Standard Solution: Pipet 5.0 ml of the 2.0 mM o-Nitrophenol Stock solution into a 100-ml volumetric flask, and dilute to volume with 1% sodium carbonate solution.

0.14 mM Standard Solution: Pipet 7.0 ml of the 2.0 mM o-Nitrophenol Stock solution into a 100-ml volumetric flask, and dilute to volume with 1% sodium carbonate solution.

0.18 mM Standard Solution: Pipet 9.0 ml of the 2.0 mM o-Nitrophenol Stock solution into a 100-ml volumetric flask, and dilute to volume with 1% sodium carbonate solution.

Substrate: Transfer 370.0 mg of *o*-nitrophenyl-β-D-galactopyranoside to a 100-ml volumetric flask, and add 50 ml of Acetate Buffer. Swirl to dissolve, and dilute to volume with Acetate Buffer.

Note: *Perform the assay procedure within 2 h of Substrate preparation.*

Test Preparation

Prepare a solution from the test sample preparation such that 1 ml of the final dilution will contain between 0.15 and 0.65 lactase unit. Weigh, and quantitatively transfer the enzyme to a volumetric flask of appropriate size. Dissolve the enzyme in water, swirling gently, and dilute with water if necessary.

Note: *Perform the assay procedure within 2 h of dissolution of the Test Preparation.*

System Suitability

Determine the absorbance of the three o-Nitrophenol Standards at 420 nm in a 1-cm cell, using a suitable spectrophotometer. Use water to zero the instrument. Calculate the millimolar extinction, M, for each of the o-Nitrophenol Standards (0.10, 0.14, and 0.18 m*M*) by the equation

$$e = A_n/C,$$

in which A_n is the absorbance of each o-Nitrophenol Standard at 420 nm and C is the corresponding concentration of *o*nitrophenol in the standard. M for each standard should be approximately 4.60/m*M*. Perform a linear regression analysis of the absorbance readings of the three o-Nitrophenol Standards versus the *o*-nitrophenol concentration in each (0.10, 0.14, and 0.18 m*M*). The r^2 should not be less than 0.99. Determine the mean M of the three o-Nitrophenol Standards for use in the calculations below.

Procedure

For each sample or blank, pipet 2.0 ml of the Substrate solution into a 25 × 150-mm test tube, and equilibrate in a water bath maintained at 37.0 ± 0.1° for approximately 10 min. At zero time, rapidly pipet 0.5 ml of the Test Preparation (or 0.5 ml of water as a blank) into the equilibrated substrate, mix by brief (1 s) vortex, and immediately return the tubes to the water bath. After exactly 15 min of incubation, rapidly add 2.5 ml of 10% sodium carbonate solution, and vortex the tube to stop the enzyme reaction. Dilute the samples and blanks to 25.0 ml by adding 20.0 ml of water, and thoroughly mix. Determine the absorbance of the diluted samples and blanks at 420 nm in a 1-cm cell, using a suitable spectrophotometer. Use water to zero the instrument.

Calculation

One lactase unit (ALU) is defined as that quantity of enzyme that will liberate *o*-nitrophenol at a rate of 1 mmol/min under the conditions of the assay. Calculate the activity (lactase activity per gram) of the enzyme preparation taken for analysis as follows:

$$ALU/g = [(A_S - B)(25)]/[(\varepsilon)(15)(W)],$$

in which,

A_S is the average of absorbance readings for the Test Preparation;
B is the average of absorbance readings for the blank;
25 is the final volume, in ml, of the diluted incubation mixture;
ε is the mean absorptivity of the o-Nitrophenol Standards per micromole**;**
15 is the incubation time, in minutes**;** and

W is the weight, in grams, of original enzyme preparation contained in the 0.5-ml aliquot of Test Preparation used in the incubation.

Glucoamylase Activity (Amyloglucosidase Activity)

Application and Principle

This procedure is used to determine the glucoamylase activity of preparations derived from *Aspergillus niger* var., but it may be modified to determine preparations derived from *Aspergillus oryzae* var. and *Rhizopus oryzae* var. (as indicated by the variations in the text below). The sample hydrolyzes *p*-nitrophenyl-α-D-glucopyranoside (PNPG) to *p*-nitrophenol (PNP) and glucose at pH 4.3 and 50°.

Use the quantity of PNP liberated per unit of time to calculate the enzyme activity. Measure the PNP liberated against a quantity of a standard preparation of PNP by measuring the absorbance of the solutions at 400 nm after adjusting the pH of the reaction mixture to pH 8.0.

Note: *Use a pH of 5.0 when testing preparations derived from Aspergillus oryzae var. or Rhizopus oryzae var.*

Apparatus

Water Bath: Use an open, circulating water bath with control accuracy of at least ± 0.1°.

Spectrophotometer: Use a spectrophotometer suitable for measuring absorbances at 400 nm.

Cuvettes: Use 10-mm light-path fused quartz.

Thermometer: Use a partial immersion thermometer with a suitable range, graduated in 1/10°.

Timer: Use a solid-state timer, model 69240 (GCS Corporation, Precision Scientific Group), or equivalent, accurate to ± 0.01 min in 240 min.

Vortex Mixer: Use a standard variable-speed mixer.

Reagents and Solutions

p-Nitrophenol Stock Solution (PNP) (0.001 M): Dissolve 139.11 mg of *p*-nitrophenol previously dried (60°, maximum 4 h) into water, and dilute to 1000 ml.

Caution: *Avoid contact with skin. If contact occurs, wash the affected area with water. Work in a well-ventilated area.*

Acetate Buffer Solution: (0.1 M) Dissolve 4.4 g of sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) in approximately 800 ml of water, add 4.5 ml of acetic acid ($C_2H_4O_2$). Adjust to pH 4.5 ± 0.05 by adding either sodium acetate or glacial acetic acid as required. Dilute to 1 l.

Note: *Use a pH of 5.0 when testing preparations derived from Aspergillus oryzae var. or Rhizopus oryzae var.*

*Sodium Carbonate Solution (0.3 M)***:** Dissolve 15.9 g of sodium carbonate (Na_2CO_3) in water, and dilute to 500 ml.

p-Nitrophenyl-α-D-glucopyranoside Solution (PNPG): Dissolve 100.0 mg of PNPG (Sigma Chemical Co., Catalog No. N1377 or equiv**a**lent) in acetate buffer, and dilute to 100 ml.

Standards

Dilute three portions of PNP Stock Solution to produce standards for the standard curve. Add 3 ml of the PNP Stock Solution to 125 ml of Sodium Carbonate Solution, and dilute to 500 ml with water to produce the first standard, containing 0.006 mmol/ml. Add 2 ml of PNP Stock Solution to 25 ml of Sodium Carbonate Solution, and dilute to 100 ml with water to produce the second standard, containing 0.02 mmol/ml. Add 5 ml of PNP Stock Solutions to 25 ml of Sodium Carbonate Solution, and dilute to 100 ml with water to produce the third standard, containing 0.05 mmol/ml.

Sample Solution

Dilute 1.00 ± 0.01 g of sample in sufficient Acetate Buffer Solution to produce a solution that contains between 0.1 and 0.3 glucoamylase units of activity per ml.

Procedure

Measure absorbances of each of the three PNP Standard Solutions to calculate the molar extinction coefficient. Equilibrate the PNPG Solution in a 50° water bath for at least 15 min. For active samples, transfer 2.0 ml of the Sample Solution to a test tube. Loosely stopper, and place the tube in the water bath to equilibrate for 5 min. At zero time, add 2.0 ml of PNPG Solution, and mix at moderate speed on a vortex mixer. Return the mixture to the water bath. Exactly 10.0 min later, add 3.0 ml of the Sodium Carbonate Solution, mix on the vortex, and remove from the water bath.

For sample blanks, transfer 2.0 ml of the Sample Solution and 3.0 ml of the Sodium Carbonate Solution into a test tube, and mix. Add 2.0 ml of PNPG Solution, and mix. Measure the absorbance of each sample and the blank versus water in a 10-mm cell.

Note: *Determine the absorbance of the sample and blank solutions not more than 20 min after adding Sodium Carbonate Solution.*

Calculations

One unit of glucoamylase activity is defined as the amount of glucoamylase that will liberate 0.1 mmol/min of p-nitrophenol from the PNPG Solution under the conditions of the assay.

Calculate the millimolar extinction of the PNP standards using the following equation:

$$\varepsilon_M = An/C,$$

in which

An is the absorbance of the p-nitrophenol standard, at 400 nm, and
C is concentration, in mmol/ml, of p-nitrophenol.
The averaged millimolar extinction coefficient, M, should be approximately 18.2.

$$\text{Glucoamylase } M = [(AS - AB) \times 7 \times F]/e_M \times 10 \times 0.10 \times W \times 2,$$

in which

AS is the sample absorbance;
AB is the blank absorbance;
F is the appropriate dilution factor;
W is the weight of sample, in grams; 7 is the final volume of the test solutions;
10 is the reaction time, in minutes; 0.10 is the amount of PNP liberated, in mmol/min/unit of enzyme;
2 is the sample aliquot, in millilitres; and
M is the millimolar extinction coefficient.

β-Glucanase Activity

Application and Principle

This procedure is used to determine β-glucanase activity of enzyme preparations derived from *Aspergillus niger* var. and *Bacillus subtilis* var. The assay is based on a 15-min hydrolysis of lichenin substrate at 40° and at pH 6.5. The increase in reducing power due to liberated reducing groups is measured by the neocuproine method.

Reagents and Solutions

Phosphate Buffer: Dissolve 13.6 g of monobasic potassium phosphate in about 1900 ml of water, add 70% sodium hydroxide solution until the pH is 6.5 ± 0.05, then transfer the solution into a 2000-ml volumetric flask, dilute to volume with water, and mix.

*Neocuproine Solution A***:** Dissolve 40.0 g of anhydrous sodium carbonate, 16.0 g of glycine, and 450 mg of cupric sulfate pentahydrate in about 600 ml of water. Transfer the solution into a 1000-ml volumetric flask, dilute to volume with water, and mix.

Neocuproine Solution B: Dissolve 600 mg of neocuproine hydrochloride in about 400 ml of water, transfer the solution into a 500-ml volumetric flask, dilute to volume with water, and mix. Discard when a yellow colour develops.

*Lichenin Substrate***:** Grind 150 mg of lichenin (Sigma Chemical Co., Catalog No. L-6133, or equivalent) to a fine powder in a mortar, and dissolve it in about 50 ml of water at about 85°. After solution is complete (20 to 30 min), add 90 mg of sodium borohydride and continue heating below the boiling point for 1 h. Add 15 g of Amberlite MB-3, or an equivalent ion-exchange resin, and stir continuously for 30 min. Filter with the aid of a vacuum through Whatman No. 1 filter paper, or equivalent, in a Buchner funnel, and wash the paper with about 20 ml of water. Add 680 mg of monobasic potassium phosphate to the filtrate, and re-filter through a 0.22-mm Millipore filter pad, or equivalent. Wash the pad with 10 ml of water, and adjust the pH of the filtrate to 6.5 ± 0.05 with 1 N sodium hydroxide or 1 N hydrochloric acid. Transfer the filtrate into a 100-ml volumetric flask, dilute to volume with water, and mix. Store at 2° to 4° for not more than 3 days.

Glucose Standard Solution: Dissolve 36.0 mg of anhydrous dextrose in Phosphate Buffer in a 1000-ml volumetric flask, dilute to volume with water, and mix.

Test Preparation

Prepare a solution from the enzyme preparation sample so that 1 ml of the final dilution will contain between 0.01 and 0.02 β-glucanase units. Weigh the sample, transfer it into a volumetric flask of appropriate size, dilute to volume with Phosphate Buffer, and mix.

Procedure

Pipet 2 ml of Lichenin Substrate into each of four separate test tubes graduated at 25 ml, and heat the tubes in a water bath at 40° for 10 to 15 min to equilibrate.

After equilibration, add 1 ml of Phosphate Buffer to tube 1 (substrate blank), 1 ml of Glucose Standard Solution to tube 2 (glucose standard), 4 ml of Neocuproine Solution *A* and 1 ml of the Test Preparation to tube 3 (enzyme blank), and 1 ml of the Test Preparation to tube 4 (sample). Prepare a fifth tube for the buffer blank, and add 3 ml of Phosphate Buffer.

Incubate the five tubes at 40° for exactly 15 min, and then add 4 ml of Neocuproine Solution A to tubes 1, 2, 4, and 5. Add 4 ml of Neocuproine Solution B to all five tubes, and cap each with a suitably sized glass marble.

Caution*: Do not use rubber stoppers.*

Heat the tubes in a vigorously boiling water bath for exactly 12 min to develop colour, then cool to room temperature in cold water, and adjust the volume of each to 25 ml with water. Cap the tubes with Parafilm, or other suitable closure, and mix by inverting several times. Determine the absorbance of each solution at 450 nm in 1-cm cells, with a suitable spectrophotometer, against the buffer blank in tube 5.

Calculation

One β-glucanase unit (BGU) is defined as that quantity of enzyme that will liberate reducing sugar (as glucose equivalent) at a rate of 1mmol/min under the conditions of the assay.

Calculate the activity of the enzyme preparation taken for analysis as follows:

$$BGU = [(A4 - A3) \times 36 \times 106]/[(A2 - A1) \times 180 \times 15 \times \text{mg sample}],$$

in which

A4 is the absorbance of the sample (tube 4),
A3 is the absorbance of the enzyme blank (tube 3),
A2 is the absorbance of the glucose standard (tube 2),
A1 is the absorbance of the substrate blank (tube 1), 36 is the micrograms of glucose in the Glucose Standard Solution,
106 is the factor converting micrograms to grams,
180 is the weight of 1 μmol of glucose, and
15 is the reaction time in minutes.

Glucose Isomerase Activity

Scope

This procedure is designed for the determination of glucose isomerase preparations derived from *Actinoplanes missouriensis, Arthrobacter globiformis, Bacillus coagulans, Streptomyces olivaceus, Streptomyces olivochromogenes,* and *Streptomyces rubiginosus*.

Principle

The assay is based on measurement of the rate of conversion of glucose to fructose in a packed bed reactor.

The procedure as outlined approximates an initial velocity assay method. Specific conditions are: glucose concentration, 45% w/w; pH (inlet) measured at room temperature in the 7.0 to 8.5 range, as specified; temperature, 60.0°; and magnesium concentration, 4 x 10^{-3} M. The optimum conditions for enzymes from different microbial sources and methods of preparation may vary; therefore, if different pH conditions, buffering systems, or methods of sample preparation are recommended by the manufacturer, such variations in the instructions given herein should be used.

Reagents and Solutions

Glucose substrate: Dissolve 539 g of anhydrous glucose and 1.0 g of magnesium sulfate, $MgSO_4 \cdot 7H_2O$, in 700 ml of water or the manufacturer's recommended buffer, previously heated to 50° to 60°. Cool the solution to room temperature, and adjust the pH as specified by the enzyme manufacturer. Transfer the solution to a 1,000-ml volumetric flask, dilute to volume with water or the specified buffer, and mix. Transfer to a vacuum flask, and de-aerate for 30 min.

Magnesium sulfate solution: Dissolve 1.0 g of magnesium sulfate, $MgSO_4 \cdot 7H_2O$, in 700 ml of water. Adjust the pH to 7.5 to 8.0 as specified by the manufacturer, using 1 N sodium hydroxide, dilute to 1,000 ml with water and mix.

Note: *Glucose isomerase activity of the commercial enzyme is usually determined on the enzyme that has been immobilized by binding with a polymer matrix or other suitable material. This method is designed for use with such preparations.*

Column Assembly and Apparatus

The column assembly is shown in Figure1 below.

Note: *Make all connections with inert tubing, glass or plastic as appropriate.*

Use a 2.5 x 40-cm glass column provided with a coarse sintered glass bottom and a water jacket connected to a constant-temperature water bath, maintained at 60.0° by means of a circulating pump. Connect the top of the column to a variable-speed peristaltic pump having a maximum flow rate of 800 ml per h. The diameter of the tubing with which the peristaltic pump is fitted should permit variation of the pumping volume from 60 to 150 ml per h. Connect the outlet of the column with a collecting vessel.

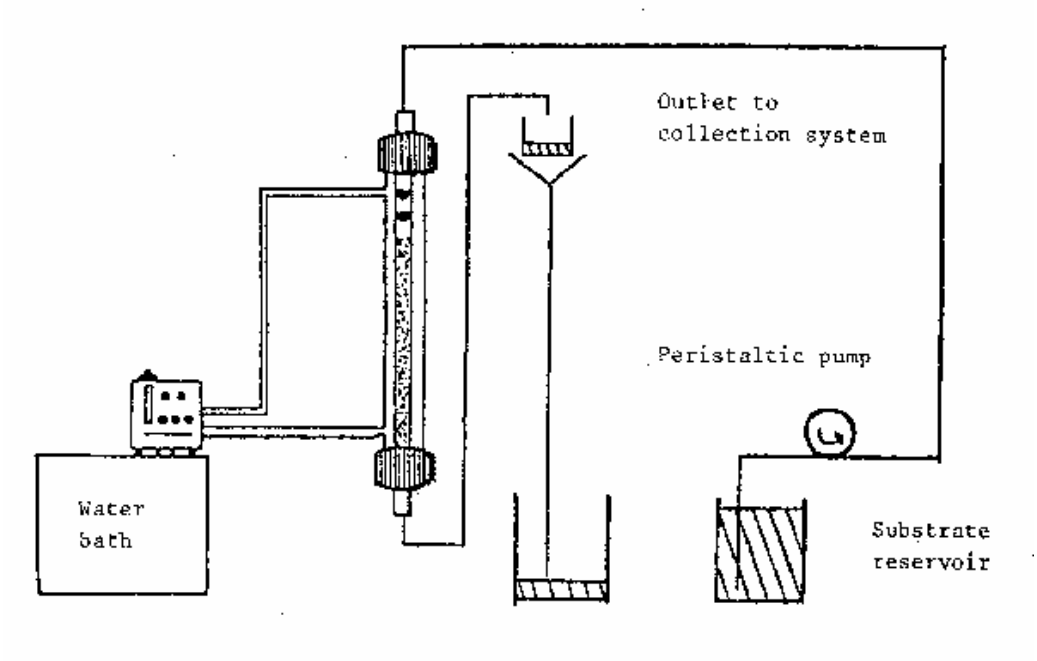


Figure 1. Diagram of a column assembly for assay of
Immobilized Glucose Isomerase

Sample Preparation

Transfer to a 500-ml vacuum flask an amount of the sample, accurately weighed in g or measured in ml, as appropriate, sufficient to obtain 2,000 to 8,000 glucose isomerase units (GI_c U). Add 200 ml of Glucose Substrate, stir gently for 15 sec and repeat the stirring every 5 min for 40 min. De-aerate by vacuum for 30 min.

Column Preparation

Quantitatively transfer the Sample Preparation to the column with the aid of Magnesium Sulfate Solution as necessary. Allow the enzyme granules to settle, and then place a porous disk so that it is even with, and in contact with, the top of the enzyme bed. All of the air

should be displaced from the disk. Place a cotton plug about 1 or 2 cm above the disk. (This plug acts as a filter. It ensures proper heating of the solution and traps dissolved gases that may be present in the Glucose Substrate.) Connect the tubing from the peristaltic pump with the top of the column, and seal the connection by suitable means in order to protect the column contents from the atmosphere. Place the inlet tube of the peristaltic pump into the Glucose Substrate solution, and begin a downward flow of the Glucose Substrate into the column at a rate of at least 80 ml per h. Maintain the flow rate for 1 h at room temperature.

Procedure

Adjust the flow of the Glucose Substrate to such a rate that a fractional conversion of 0.2 to 0.3 will be produced, based on the estimated activity of the sample. The fractional conversion is calculated from optical rotation values obtained on the starting Glucose Substrate and the sample effluent, as specified in Calculations below. After the correct flow rate has been established, run the column overnight (16 h minimum), then check the pH of the Glucose Substrate, and readjust if necessary to the specified pH. Measure the flow rate, and collect a sample of the column effluent. Cover the effluent sample, allow it to stand for 30 min at room temperature, and then determine the fractional conversion of glucose to fructose (see Calculations below). If the conversion is less than 0.2 or more than 0.3, adjust the flow rate to bring the conversion into this range. If a flow rate adjustment is required, collect an additional effluent sample after allowing the column to re-equilibrate for at least 2 h and then determine the fractional conversion.

Measure the flow rate, and collect an effluent sample. Cover the sample, let it stand at room temperature for 30 min, and determine the fractional conversion.

Calculations

Specific rotation

Measure the optical rotation of the effluent sample and of the starting Glucose Substrate at 25.0°, and calculate their specific rotations by the formula:

$$[\alpha]^{25}_{D} = 100\ a/lpd$$

in which

a is the corrected observed rotation, in degrees,
l is the length of the polarimeter tube, in dm,
p is the concentration of the test solution, expressed as g of solute per 100 g of solution, and
d is the specific gravity of the solution at 25°

Fractional conversion

Calculate the fractional conversion, X, by the formula:

$$X = (\alpha_E - \alpha_S) / (\alpha_F - \alpha_S)$$

in which

α_E is the specific rotation of the column effluent,
α_S is the specific rotation of the Glucose Substrate,
and α_F is the specific rotation of fructose (which in this case has been calculated to be -94.54).

Activity

The enzyme activity is expressed in glucose isomerase units (CI_cU, the subscript c signifying column process). One GI_cU is defined as the amount of enzyme that converts glucose to fructose at an initial rate of 1 µmol per min, under the conditions specified.

Calculate the glucose isomerase activity by the formula:

$$CI_cU \text{ per g or ml} = (FS/W)\,[X_e \ln X_e / (X_e - X)]$$

in which

F is the flow rate, in ml per min,
S is the concentration of the Glucose Substrate, in µml per ml,
X_e is the fractional conversion at equilibrium, or 0.51, and
W is the weight or volume of the sample taken, in g or ml, respectively.

Glucose Oxidase Activity

Application and Principle

This procedure is used to determine glucose oxidase activity in preparations derived from *Aspergillus niger* var. The assay is based on the titrimetric measurement of gluconic acid produced in the presence of excess substrate and excess air.

Reagents and Solutions

Chloride–Acetate Buffer Solution: Dissolve 2.92 g of sodium chloride and 4.10 g of sodium acetate in about 900 ml of water. Adjust the pH to 5.1 with either dilute acetic acid or dilute sodium hydroxide solution and dilute to 1000.0 ml.

Sodium Hydroxide Solution (0.1 N)

Hydrochloric Acid Solution (0.05 N) Standardized.

Phenolphthalein Solution (2% w/v): Solution in methanol.

Octadecanol Solution: Saturated solution in methanol.

Substrate Solution: Dissolve 30.00 g of anhydrous glucose in 1000 ml of the Chloride–Acetate Buffer Solution.

Sample Preparation

Dissolve an accurately weighed amount of enzyme preparation in the Chloride–Acetate Buffer Solution, and dilute in the buffer solution to obtain an enzyme activity of 5 to 7 activity units per milliliter.

Procedure

Transfer 25.0 ml of the Substrate Solution to a 32 × 200-mm test tube. To a second 32 × 200 mm test tube transfer 25.0 ml of the Chloride–Acetate Buffer Solution (blank). Equilibrate both tubes in a 35 ± 0.1° water bath for 20 min. Add 3.0 ml of the Sample Preparation to each test tube, mix, and insert a glass sparger into each tube with a pre-adjusted air flow of 700 to 750 ml/min. If excessive foaming occurs, add 3 drops of the Octadecanol Solution to each tube. After exactly 15 min, remove the sparge and rinse any adhering reaction mixture back into the tube with water. Immediately add 10 ml of the Sodium Hydroxide Solution and 3 drops of the Phenolphthalein Solution to each tube. Insert a small magnetic stirrer bar, stir, and titrate to the phenolphthalein endpoint with the standardized 0.05 N hydrochloric Acid Solution.

Calculation

One Glucose Oxidase Titrimetric unit of activity (GOTu) is the quantity of enzyme that will oxidize 3 mg of glucose to gluconic acid under the conditions of the assay. Determine the enzyme activity using the following equation:

$$GOTu/g = [(B - T) \times N \times 180 \times F]/[3 \times W],$$

in which

B is the titration volume, in milliliters, of the blank;
T is the titration volume, in milliliters, of the sample;
N is the normality of the titrant; 180 is the molecular weight of glucose;
F is the sample dilution factor;
3 is from the unit definition; and
W is the weight, in grams, of the enzyme preparation contained in each milliliter of the sample solution.

Glutaraldehyde Limit Test

Scope

This procedure is designed to determine the glutaraldehyde carried over into isomerized syrup during isomerization of glucose syrup by the use of immobilized glucose isomerases crosslinked with glutaraldehyde.

Principle

The procedure involves sampling the syrup produced during different stages of the enzyme assay "Glucose isomerase activity". Analysis of the sample syrup according to the procedure on page 169 gives the number of mg of glutaraldehyde per kg of syrup. A subsequent calculation gives the amount of glutaraldehyde present per unit of glucose isomerase activity. The enzyme preparation passes the test if the average result is not greater than 0.025.

Procedure

Samples of syrup during the assay for "Glucose isomerase activity" are taken at steps as prescribed in the following:

Sample 1: 25 ml of syrup is taken out at the step called "Sample preparation" (i.e. syrup decanted off, just after the prescribed 40 min soaking period).

Sample 2: 25 ml of syrup is taken out at the step called "Procedure" (i.e. isomerized syrup from the column outlet just after the flow rate has been adjusted to the correct level).

Sample 3: 25 ml of syrup is taken out at the point of time when samples are taken for determination of the fractional conversion of the glucose to fructose.

As prescribed, this time is at least 16 hours after start-up. In actual practice the time for taking this effluent sample will be in the interval 42-48 hours after start-up.

All three samples (Samples 1, 2, and 3) are subjected to determination for glutaraldehyde as described in "Determination of glutaraldehyde in High Fructose Corn Syrup". As indicated in the text of the assay, it has been determined that the lower detection limit for glutaraldehyde in HFCS (High Fructose Corn Syrup) is 5 mg/kg by this assay.

Calculation

The relationship between the determination of glutaraldehyde and the determination of activity of the prepared immobilized enzyme can be expressed in the following way:

a = (mg GA/kg syrup) / (GI_cU/g enzyme)

in which

GA is Glutaraldehyde
GI_cU is the activity unit for glucose isomerase in the column process

Interpretation of test results

The enzyme passes test if the average "a" from the three samples tested is not greater than 0.025. (For GA concentrations below the detection limit of 5 mg/kg, the value 5 mg/kg is taken.)

Examples

- a = 0.025 is equal to an average GA concentration of 5 mg/kg from 200 GI_cU/g enzyme.
- a = 0.025 is equal to an average GA concentration of 7.5 mg/kg from 300 GI_cU/g enzyme.

Glutaraldehyde Determination in High Fructose Corn Syrup (High Fructose Glucose Syrup)

Scope

This procedure is designed for the determination of Glutaraldehyde in High Fructose Corn Syrup (HFCS).

Principle

The assay is based on a measurement using thin layer chromatography.

Apparatus

TLC plates: Pre-coated TLC plates SIL G-25, available from Macherey-Nagel, Catalog No. 809 013, or equivalent. Activate before use by heating to 100° for at least one h. Use gloves when handling.

Reagents

Solvent system: Transfer 5.0 ml absolute ethanol to a 100-ml volumetric flask and fill up to the mark with chloroform. Transfer to a 250-ml flask and shake very thoroughly before pouring the mixture into the developing chamber.

Spray reagents: (Sufficient for one TLC plate)

I: *1% MBTH*: Dissolve 250 mg MBTH (N-methyl-benzothiazolonhydrazon-HCl) in 25 ml water.

II: *2% Ferric chloride*: Dissolve 0.5 g ferric chloride ($FeCl_3 \cdot 6H_2O$) in 25 ml water.

Standard Solutions

Glutaraldehyde stock solution (1 mg/ml): Transfer 0.4 ml of 25% glutardialdehyde solution (Merck No. 12179) to a 100-ml volumetric flask. Make up to the mark with water.

Glutaraldehyde solution (25 µg/ml): Dilute 250 µl of glutaraldehyde stock solution to 10.0 ml with water. Dilution to be made freshly before use.

Glutaraldehyde solution (3.75 µg/ml): Dilute 1.50 ml of G - 25 µg/ml to 10.0 ml with water. Dilution to be made freshly before use.

Assay Solutions

Transfer to 10-ml volumetric flasks:

Assay solution (a): 7.50 g of HFCS sample;

Assay solution (b): 7.50 g of HFCS sample and 1.50 ml of glutaraldehyde solution (25 µg/ml) corresponding to 37.5 µg of glutaraldehyde.

Make both solutions up to volume with water.

Procedure

Treat the standard and assay solutions for 30 min in an ultra-sonic bath immediately before use.

Spot the TLC plate as follows:

- Spot 1: 150 µl of glutaraldehyde solution (3.75 µg/ml) equivalent to 0.5625 µg glutaraldehyde.
- Spot 2: 150 µl of assay solution (b) equivalent to 0.5625 µg glutaraldehyde plus 0.1125 g HFCS sample.
- Spot 3: 150 µl of assay solution (a) equivalent to 0.1125 g HFCS sample.

The spots should be placed at least 3 cm from the edges of the plate and 5 cm apart. Allow the spots to dry at room temperature. Run the chromatogram until the solvent front has migrated 15 cm (30-40 min). Allow the plate to dry for at least 30 min at room temperature.

Spray with reagent I using a fine nozzle. Approximately 20 ml are needed.

Wait for 10 min and then spray with reagent II until the spots can be seen. Approximately 25 ml are needed.

Estimation

Estimate the glutaraldehyde content of the assay sample (spot 3) by comparison with the standard (spot 1).

If the intensity of assay sample spot 3 is less than the intensity of standard spot 1, then the HFCS sample contains < 5 mg/kg of glutaraldehyde.

Spot 2 is included as proof that the method can detect 5 mg/kg of glutaraldehyde in HFCS.

Hemicellulase Activity

Scope

This procedure is for the determination of hemicellulase activity of preparations derived from *Aspergillus niger*, var.

Principle

The test is based on the enzymatic hydrolysis of the interior glucosidic bonds of a defined carob (locust) bean gum substrate at pH 4.5 and 40°. The corresponding reduction in substrate viscosity is determined with a calibrated viscometer.

Apparatus

Viscometer: Use a size 100 calibrated Cannon-Fenske Type Viscometer, or its equivalent. A suitable viscometer is supplied as Catalog No. 2885-100 by Scientific Products, 1210 Waukegan Road, McGraw Park, Ill. 60085.

Glass Water Bath: Use a constant-temperature glass water bath maintained at 40 ± 0.1°. A suitable bath is supplied as Catalog No. W3520 10 by Scientific Products.

Reagents and Solutions

Acetate Buffer (pH 4.5): Add 0.2 N sodium acetate, with continuous agitation, to 400 ml of 0.2 N acetic acid until the pH is 4.5 ± 0.05, as determined by a pH meter.

Locust Bean Gum: Use Powdered Type D-200 locust bean gum, or its equivalent, supplied by Meer Corp., 9500 Railroad Avenue, North Bergen, N.J. 07047. Since the substrate may vary from lot to lot, each lot should be tested in parallel with a previous lot known to be satisfactory. Variations of more than ± 5% viscosity in the average of a series of parallel tests indicate an unsuitable lot.

Substrate Solution: Place 12.5 ml of 0.2 N hydrochloric acid and 250 ml of warm water (72° to 75°) in the bowl of a power blender (Waring two-speed, or its equivalent, supplied as Catalog No. 58350-1 by Scientific Products), and set the blender on low speed. Slowly disperse 2.0 g of Locust Bean Gum, on a moisture-free basis, into the bowl, taking care not to splash out any of the liquid in the bowl. Wash down the sides of the bowl with warm water, using a rubber policeman, cover the bowl, and blend at high speed for 5 min. Quantitatively transfer the mixture to a 1,000-ml beaker, and cool to room temperature. Using a pH meter, adjust the mixture to pH 6.0 with 0.2 N sodium hydroxide. Quantitatively transfer to a 1,000-ml volumetric flask, dilute to volume with water, and mix. Filter the substrate through gauze before use.

Sample Preparation

Prepare a solution of the sample in water so that 1 ml of the final dilution will produce a change in relative fluidity between 0.18 and 0.22 in 5 min under the conditions specified in the *Procedure* below. Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with water. Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute to volume with water, and mix. Filter through Whatman No. 1 filter paper, or equivalent, before use.

Procedure

Scrupulously clean the Viscometer by drawing a large volume of detergent solution, followed by water, through the instrument, and place the viscometer, previously calibrated, in the Glass Water Bath in an exactly vertical position. Pipet 20.0 ml of Substrate Solution and 4.0 ml of Acetate Buffer into a 50-ml Erlenmeyer flask, allowing at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks, and equilibrate them in the water bath for 15 min. At zero time, pipet 1.0 ml of the Sample Preparation into the equilibrated substrate, start timing with a stopwatch (No. 1), and mix thoroughly. Immediately pipet 10.0 ml of this mixture into the wide arm of the Viscometer. After about 2 min, draw the reaction mixture above the upper mark into the driving fluid head by applying suction with a rubber tube connected to the narrow arm of the instrument. Measure the efflux time by allowing the reaction mixture to flow freely down past the upper mark. As the meniscus falls past the upper mark, start the second stopwatch (No. 2), and at the same time record the reaction time (T_R), in min, from stopwatch No. 1. As the meniscus of the reaction mixture falls past the lower mark, record the time (T_T), in sec, from stopwatch No. 2.

Immediately re-draw the reaction mixture above the upper mark and into the driving fluid head. As the meniscus falls freely past the upper mark, restart stopwatch No. 2, and at the same time record the reaction time (T_R), in min, from stopwatch No. 1. As the meniscus falls past the lower mark, record the time (T_T), in sec, from stopwatch No. 2. Repeat the latter operation, beginning with "Immediately re-draw the reaction mixture ..." until a total of four determinations are obtained over a reaction time (T_R) of not more than 15 min.

Prepare a substrate blank by pipetting 1.0 ml of water into a mixture of 20.0 ml of Substrate Solution and 4.0 ml of Acetate Buffer, and then immediately pipet 10.0 ml of this mixture into the wide arm of the Viscometer. Determine the time (T_S), in sec, required for the meniscus to fall between the two marks. Use an average of five determinations as T_S.

Prepare a water blank by pipetting 10.0 ml of water, previously equilibrated to 40 ± 0.1°, into the wide arm of the Viscometer. Determine the time (T_w), in sec, required for the meniscus to fall between the two marks. Use an average of five determinations as T_w.

Calculation

One hemicellulase unit (HCU) is that activity that will produce a relative fluidity change of 1 over a period of 5 min in a locust bean gum substrate under the conditions specified. Calculate the relative fluidities (FR) and T values (see definition below) for each of the four efflux times (T_T) and reaction times (T_R) as follows:

$$F_R = (T_S - T_W)/(T_T - T_w),$$

and

$$T_N = 1/2(T_T/60) + T_R = (T_T/120) + T_R,$$

in which

F_R is the relative fluidity for each reaction time;
T_S is the average efflux time for the substrate blank, in sec;
T_w is the average efflux time for the water blank, in sec;
T_T is the efflux time of the sample reaction mixture, in sec;
T_R is the elapsed time from zero time, in min, i.e., the time from addition of the enzyme solution to the buffered substrate, until the beginning of the measurement of the efflux time (T_T); and
T_N is the reaction time (T_R), in min, plus one half of the efflux time (T_T) converted to min.

Plot the four relative fluidities (F_R) as the ordinate against the four reaction times (T_N) as the abscissa. A straight line should be obtained. The slope of the line corresponds to the relative fluidity change per min and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a single relative fluidity value. From the curve determine the F_R values at 10 and 5 min. They should have a difference in fluidity of not more than 0.22 and not less than 0.18. Calculate the activity of the enzyme sample as follows:

$$HCU/g = 1{,}000(F_{R10} - F_{R5})/W,$$

in which

F_{R10} is the relative fluidity at 10 min reaction time;
F_{R5} is the relative fluidity at 5 min reaction time;
1,000 is mg per g; and
W is the weight, in mg, of the enzyme sample contained in the 1.0-ml aliquot of Sample Preparation added to the equilibrated substrate in the Procedure.

Invertase Activity

Principle

Invertase hydrolyses the non-reducing β-d-fructofuranoside residues of sucrose to yield invert sugar. The invert sugar released is then reacted with 3.5 dinitrosalicylic acid (DNS). The colour change produced is proportional to the amount of invert sugar released, which in turn is proportional to the invertase activity present in the sample. The absorbance is measured at 540 nm and converted into micromoles of reducing sugar produced using a standard curve. One invertase unit is the amount of enzyme which will produce 1 micromole of reducing sugar (expressed as invert sugar) per minute under the conditions specified in this procedure.

Apparatus

Spectrophotometer set at 540 nm
Water bath set at 30±1.0°
Stopwatch
Boiling water bath
Ice water bath
Mixer

Reagents and solutions

0.05 M Sodium acetate buffer, pH 4.7: Adjust the pH of 200 ml of 0.05 M sodium acetate (4.1 g of sodium acetate anhydrous in 1000 ml of water) to pH 4.7 ± 0.05 with 0.05M acetic acid (2.85 ml of glacial acid in 1000 ml of water).

0.3 M Sucrose: 5.13 g sucrose in 50.0 ml of water

20 mM Tris HCl buffer, pH 7.0: Dissolve 2.42 g of tris (hydroxymethyl) aminomethane in about 800 ml of water. Adjust pH to 7.0 using 5% hydrochloric acid (5 ml of conc. hydrochloric acid in 100.0 ml of water).

DNS solution: Weigh 300 g of potassium sodium tartrate tetrahydrate into a one litre conical flask. Add 16 g of sodium hydroxide and 500 ml of water and dissolve by heating gently. When the solution is clear, add slowly 10 g of 3,5-dinitrosalicylic acid (DNS). Keep covered to protect from light until the DNS is totally dissolved. Cool to room temperature and make up to 1 litre with water. Store in a tightly stoppered dark container. Protect from light and carbon dioxide.

Invert sugar standard (0.01M): Dry glucose to constant weight at 105° and dry fructose to constant weight at 70° under vacuum. Dissolve 0.9 g of glucose and 0.9 g of fructose in 1000 ml of 0.1% benzoic acid (1 g of benzoic acid in 1000 ml of water).

Standard curve

Prepare a series of test tubes, in duplicate, according to the table below. The standard curve must include at least four suitable standards

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**Blank**
Invert sugar standard (ml)	0.1	0.3	0.5	0.8	1.0	1.2	0.0
Water (ml)	2.4	2.2	2.0	1.7	1.5	1.3	2.5
Acetate buffer (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Content of invert sugar	1.0	3.0	5.0	8.0	10	12	0.0

Reaction and measurement

Mix and incubate for exactly 10 min at 30 ± 0.1°. Add 2.0 ml of DNS solution to each tube, cover tubes and place all tubes in a boiling water bath for exactly 10 min. Cool rapidly in an ice water bath and add 15 ml of water to each tube. Mix thoroughly. Measure the absorbance at 540 nm of each sample using the blank to zero the spectrophotometer. Plot the absorbance against content of invert sugar.

Sample preparation

Accurately weigh about 1 g of the sample and dissolve in 10 ml of 20 mM Tris HCl buffer. For powder samples it may be necessary to use a magnetic stirrer for up to 10 min. Dilute the sample with 20 mM Tris HCl buffer to obtain a solution for which the measured absorbance will fall within the linear range of 0.14 and 0.30.

Procedure

Into each of a series of 30 ml test tubes, pipette, in quadruplicate, 1.4 ml of water, 0.5 ml of acetate buffer and 0.1 ml of diluted enzyme. Equilibrate the tubes in a 30° water bath. Add 1 ml of 0.3 M sucrose solution to 3 of the 4 tubes. Use the fourth tube as an enzyme blank, adding 2 ml of DNS solution before adding 1.0 ml of 0.3 M sucrose solution. Prepare a reagent blank using 0.1ml of water in place of diluted enzyme. Continue as described under 'Reaction and measurement'. Read the respective contents of invert sugar from the standard curve.

Calculation

$$\text{Activity for powders (units/minute/g)} = [(C_S - C_B) \times \text{dilution}] / W$$

Where

C_S is Content of invert sugar in sample solution (micromoles)
C_B is Content of invert sugar in enzyme blank solution (micromoles)
W is Weight of sample (g)

$$\text{Activity for liquids (units/minute/ml)} = [(C_S - C_B) \times \text{dilution} \times \text{S.G.}] / W$$

Where

C_S is Content of invert sugar in sample solution (micromoles)
C_B is Content of invert sugar in enzyme blank solution (micromoles)
W is Weight of sample (g)
S.G. is Specific gravity of sample (g/ml)

Milk Clotting Activity

Scope

This procedure is designed to be applied to enzyme preparations derived from either animal or microbial sources.

Principle

The method is based on a visual flocculation endpoint.

Apparatus

Bottle-rotating apparatus: Use a suitable assembly, designed to rotate at a rate of 16 to 18 rpm, such as the Dries-Jacques Associates type model (Available from Dries-Jacques Associates, 1801 East North Avenue, Milwaukee, Wisconsin 53202, USA.) or equivalent

Sample bottles: Use 125-ml squat, round, wide-mouth bottles such as those available as Catalog No. 2-903 from Fisher Scientific Co. (Available from Fischer Scientific, 711 Forbes Av., Pittsburgh, PA 15219, USA.), or equivalent.

Reagents

Substrate Solution: Dissolve 60 g of low-heat, non-fat dry milk (such as Peake Grade A (Available from Galloway West, Fond du Lac, Wisc. 54935, USA.)), or equivalent in 500 ml of a solution, adjusted to pH 6.3 if necessary, containing in each ml 2.05 mg of sodium acetate ($NaC_2H_3O_2$) and 1.11 mg of calcium chloride ($CaCl_2$).

Standard Preparation: Use a standard-strength rennet; bovine rennet; milk-clotting enzyme, microbial (*E. parasitica*); or milk-clotting enzyme, microbial (Mucor species) as appropriate for the preparation to be assayed. Such standards, which are available from commercial coagulant manufacturers, should be of known activity. Dilute the standard-strength material 1 to 200 with water, and mix. Equilibrate to 30° before use, and prepare no more than 2 h prior to use.

Sample Preparation

Prepare aqueous solutions or dilutions of the sample to produce a final concentration such that the clotting time, as determined in the Procedure below, will be within 1 min of that of the Standard Preparation. Prepare no more than 1 h prior to use.

Procedure

Transfer 50.0 ml of the Substrate Solution into each of four 125-ml Sample Bottles. Place the bottles on the Bottle-rotating Apparatus, and suspend the apparatus in a water bath, maintained at 30° ± 0.5, so that the bottles are at an angle or approximately 20° to 30° to the horizontal. Immerse the bottles so that the water level in the bath is about equal to the substrate level in the bottles. Begin rotating the apparatus at 16-18 rpm, then add 1.0 ml of the Sample Preparation to each of the two bottles, and record the exact time of addition. Add 1.0 ml of the Standard Preparation to each of the other two bottles, recording the exact time. Observe the rotating bottles, and record the exact time of the first evidence of clotting (i.e. when fine granules or flecks adhere to the sides of the bottle). Variations in the response of different lots of the substrate may cause variations in clotting time; therefore, the test samples and standards should be measured simultaneously on the same substrate. Average the clotting time, in sec, of the duplicate samples, recording the time for the Standard Preparation as T_s and that for the Sample Preparation as T_v.

Calculation

Calculate the activity of the enzyme preparation by the formula:

$$\text{Milk-clotting Units/ml} = 100 \times (T_s/T_v) \times (D_s/D_v)$$

in which 100 is the activity assigned to the Standard Preparation, D_s is the dilution factor for the Standard Preparation, and D_v is the dilution factor for the Sample Preparation.

Note: *The dilution factors should be expressed as fractions; e.g., a dilution of 1 to 200 should be expressed as 1/200.*

Protease Activity (Viscometer method)

Scope

This procedure is designed for the determination of protease activity at pH 7.

Principle

This assay is based on the enzymatic hydrolysis of the peptide bonds of a defined gelatin substrate at pH 7.0 and 40°. The corresponding reduction in substrate viscosity is determined with a calibrated viscometer. One Viscometric Protease Unit is defined as that activity which will produce a relative fluidity change of 0.01 per sec in a defined gelatine substrate under the conditions of the assay.

Special Apparatus

Calibrated viscometer: Size 100 Calibrated Cannon-Fenske Type Viscometer, or its equivalent, supplied as Catalog No. P2885-100.

Constant temperature glass water bath (40 ± 0.1°): Constant temperature glass water bath, or its equivalent, supplied as Catalog No. W3520-10 (Available from Scientific Products, 1210 Waukegan Rd., McGaw Park, Ill., 60085, USA.).

Stopwatches: Stopwatch calibrated in 1/10 min for determining the reaction time (T_r) and stopwatch calibrated in 1/5 sec for determining the efflux time (T_t).

Reagents and Solutions

Disodium monohydrogen phosphate solution (1 N): Dissolve 47.32 g of anhydrous disodium phosphate in approximately 800 ml of distilled water in a beaker. Quantitatively transfer to a 1,000-ml volumetric flask and dilute to volume with distilled water.

Monosodium dihydrogen phosphate solution (1 N): Dissolve 40.00 g of anhydrous monosodium phosphate in approximately 800 ml of distilled water in a beaker. Quantitatively transfer to a 1,000-ml volumetric flask and dilute to volume with distilled water.

Phosphate buffer (pH 7.0): Using a standardized pH-meter, add disodium monohydrogen phosphate solution (1 N) with continuous agitation to 800 ml of monosodium dihydrogen phosphate solution (1 N) until the buffer is pH 7.0 ± 0.05.

Gelatine substrate (4.0% w/v): With continuous agitation, disperse 20.00 g (moisture-free basis) of gelatin in approximately 400 ml of distilled water in a 1,000-ml Erlenmeyer flask. The dispersion must be free of lumps. Swell the gelatin for 30 min at room temperature with occasional swirling. Place the gelatin solution on a 40 ± 0.1° waterbath. Swirl occasionally until the gelatin is completely solubilized with no particles appearing in solution. Cool to room temperature and quantitatively transfer to a 500-ml volumetric flask and dilute to volume with distilled water.

Enzyme Preparation: Prepare an enzyme solution so that 1 ml of the final dilution will produce a relative fluidity change between 0.18 and 0.22 in 5 min under the conditions of the assay. Weigh the enzyme and quantitatively transfer to a glass mortar. Triturate the enzyme with distilled water and quantitatively transfer to an appropriate volumetric flask. Dilute the volume with distilled water and filter the enzyme solution through Whatman No. 1 filter paper, or equivalent, prior to use.

Procedure

Place the calibrated viscometer in the 40 ± 0.1° water bath in an exactly vertical position. Use only a clean viscometer. Cleaning is readily accomplished by drawing a large volume of detergent solution followed by distilled water through the viscometer. This can be accomplished by using an aspirator with a rubber tube connected to the narrow arm of the viscometer.

Pipet 20 ml of gelatin substrate and 3 ml of phosphate buffer into a 50-ml Erlenmeyer flask. Allow at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks and equilibrate them in the water bath for 15 min. At zero time pipet 1 ml of the enzyme solution into the equilibrated substrate. Start the stopwatch calibrated in 0.1 min and mix solution thoroughly. Immediately pipet 10 ml of the reaction mixture into the wide arm of the viscometer.

After approximately 2 min apply suction with a rubber tube connected to the narrow arm of the viscometer drawing the reaction mixture above the upper mark into the driving fluid head. Measure the efflux time by allowing the reaction mixture to freely flow down past the upper mark. As the meniscus of the reaction mixture falls past the upper mark, start the other stopwatch. At the same time record the reaction time in min from the first stopwatch (T_r). As the meniscus of the reaction mixture falls past the lower mark, record the time in sec from the second stopwatch (T_t). Immediately redraw the reaction mixture above the upper mark and into the fluid driving head. As the meniscus of the reaction mixture falls freely past the upper mark, restart the second stopwatch. At the same time, record the reaction time in min from the first stopwatch (T_r). As the meniscus of the reaction mixture falls past the lower mark, record the time in sec, from the second stopwatch (T_t).

Repeat from redrawing the reaction mixture above the upper mark, until a total of 4 determinations is obtained over a reaction time (T_r) of not more than 15 min.

Prepare a substrate blank by pipetting 1 ml of distilled water into 24 ml of buffered substrate. Pipet 10 ml of the reaction mixture into the wide arm of the viscometer. Determine the time (T_s) in sec required for the meniscus to fall between the two marks. Use an average of 5 determinations for T_s.

Prepare a water blank by pipetting 10 ml of equilibrated distilled water into the wide arm of the viscometer. Determine the time (T_w) in sec required for the meniscus to fall between the two marks. Use an average of 5 determinations for T_w.

Calculation

One Viscometric Protease Unit (VPU) is that activity which will produce a relative fluidity change of 0.01 per sec in a defined gelatin substrate under the conditions of the assay.

Calculate the relative fluidities (F_r) and the times (T_n) for each of the four (4) efflux times (T_t) and reaction times (T_r) as follows:

$$F_r = (T_s - T_w)/(T_t - T_w)$$

$$T_n = 1/2\ (T_t/60) + T_r = (T_t/120) + T_r$$

where

F_r is relative fluidity for each reaction time,
T_s is average efflux time for the substrate blank in sec,
T_w is average efflux time for the water blank in sec,
T_t is efflux time of the reaction mixture in sec,
T_r is elapsed time in min from zero time, i.e. the time from addition of the enzyme solution to the buffered substrate, until the beginning of the measurement of efflux time (T_t),
T_n is reaction time in min (T_r), plus one-half of the efflux time (T_t) converted to min.

Plot the four relative fluidities (F_r) as the ordinate against the four reaction times (T_r) as the abscissa. A straight line should be obtained. The slope of this line corresponds to the relative fluidity change per min and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a

single relative fluidity value. From the graph determine the F_r values at 10 and 5 min. They should have a difference in fluidity of not more than 0.22 nor less than 0.18. Calculate the activity of the enzyme unknown as follows:

$$VPU/g = [1{,}000 (F_{r10} - F_{r5})] / (W \times 300 \times 0.01) = [333 (F_{r10} - F_{r5})] / W$$

where

F_{r5} is relative fluidity at five (5) min of reaction time
F_{r10} is relative fluidity at ten (10) min of reaction time
300 is time of relative fluidity change in sec from F_{r10} to F_{r5}
1,000 is milligrams per g
W is weight in milligrams of enzyme added to the reaction mixture in a one (1) ml aliquot of enzyme solution
0.01 is change in relative fluidity per sec per VPU.

Proteolytic Activity, Bacterial (PC)

Scope

This procedure is designed for the determination of protease activity, expressed as PC units.

Principle

The assay is based on a 30-min proteolytic hydrolysis of casein at 37° and pH 7.0. Unhydrolyzed casein is removed by filtration, and the solubilized casein is determined spectrophotometrically.

Reagents and Solutions

Casein: Use Hammarsten-grade casein (Available from Nutritional Biochemical Corp., 21010 Miles Ave., Cleveland, Ohio 44128, USA.) or equivalent.

Tris buffer (pH 7.0): Dissolve 12.1 g of enzyme-grade (or equivalent) tris(hydroxymethyl)aminomethane in 800 ml of water, and titrate with 1 N hydrochloric acid to pH 7.0. Transfer into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

TCA solution: Dissolve 18 g of trichloroacetic acid and 19 g of sodium acetate trihydrate in 800 ml of water in a 1,000-ml volumetric flask, add 20 ml of glacial acetic acid, dilute to volume with water, and mix.

Substrate solution: Dissolve 6.05 g of tris(hydroxymethyl)aminomethane (enzyme grade) in 500 ml of water, add 8 ml of 1 N hydrochloric acid, and mix. Dissolve 7 g of Casein in this solution, and heat for 30 min in a boiling water bath, stirring occasionally. Cool to room temperature, and adjust to pH 7.0 with 0.2 N hydrochloric acid, adding the acid slowly, with vigorous stirring, to prevent precipitation of the casein. Transfer the mixture into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

Sample Preparation

Using Tris Buffer, prepare a solution of the sample enzyme preparation so that 2 ml of the final dilution will contain between 10 and 44 PC units.

Procedure

Pipet 10.0 ml of the Substrate Solution into each of a series of 25 x 150-mm test tubes, allowing one tube for each enzyme test, one tube for each enzyme blank, and one tube for a substrate blank. Equilibrate the tubes for 15 min in a water bath maintained at 37 ± 0.1°. At zero time, rapidly pipet 2.0 ml of the Sample Preparation into the equilibrated substrate,

starting the stopwatch at zero time. Mix, and replace the tubes in the water bath. Add 2 ml of Tris Buffer (instead of the Sample Preparation) to the substrate blank.

After exactly 30 min, add 10 ml of TCA Solution to each enzyme incubation and to the substrate blank to stop the reaction. **Caution:** *Do not use mouth suction for the TCA Solution*. Heat the tubes in the water bath for an additional 30 min to allow the protein to coagulate completely.

At the end of the second heating period, shake each tube vigorously, and filter through 11-cm Whatman No. 42, or equivalent, filter paper, discarding the first 3 ml of filtrate.

Note: *The filtrate must be perfectly clear.*

Determine the absorbance of each sample filtrate in a 1-cm cell, at 275 nm, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument at zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained in A_u.

Standard Curve

Transfer 100 mg of L-tyrosine, chromatographic-grade (Available from Calbiochem, La Jolla, Calif. 92037, USA.) or equivalent, previously dried to constant weight, to a 1,000-ml volumetric flask. Dissolve in 60 ml of 0.1 N hydrochloric acid.

When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 100 µg of tyrosine in 1.0 ml. Prepare three more dilutions from this stock solution to contain 75.0, 50.0 and 25.0 µg of tyrosine per ml. Determine the absorbance of the four solutions at 275 nm in a 1-cm cell with a suitable spectrophotometer versus 0.006 N hydrochloric acid. Prepare a plot of absorbance versus tyrosine concentration.

Calculation

One bacterial protease unit (PC) is defined as that quantity of enzyme that produces the equivalent of 1.5 µg per ml of L-tyrosine per min under the conditions of the assay.

From the Standard Curve, and by interpolation, determine the absorbance of a solution having a tyrosine concentration of 60 µg per ml. A figure close to 0.0115 should be obtained. Divide the interpolated value by 40 to obtain the absorbance equivalent to that of a solution having a tyrosine concentration of 1.5 µg per ml and record the value thus derived as A_s.

Calculate the activity of the sample enzyme preparation by the formula:

$$PC/g = (A_u/A_s) \times (22/30W)$$

in which

22 is the final volume, in ml of the reaction mixture,
30 is the time of the reaction, in min, and
W is the weight of the original sample taken, in g.

Proteolytic Activity, Fungal (HUT)

Scope

This procedure is for the determination of the proteolytic activity, expressed as haemoglobin units on the tyrosine basis (HUT), of preparations derived from *Aspergillus oryzae*, var., and *Aspergillus niger*, var., and it may be used to determine the activity of other proteases at pH 4.7.

Principle

The test is based on the 30-min enzymatic hydrolysis of a haemoglobin substrate at pH 4.7 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized haemoglobin in the filtrate is determined spectrophotometrically.

Reagents and Solutions

Haemoglobin: Use Haemoglobin Substrate Powder (Sigma Chemicals Co., Catalog No. H 262) or a similar high-grade material that is completely soluble in water.

Acetate Buffer Solution: Dissolve 136 g of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) in sufficient water to make 500 ml. Mix 25.0 ml of this solution with 50.0 ml of 1 M acetic acid, dilute to 1,000 ml with water, and mix. The pH of this solution should be 4.7 ± 0.02.

Substrate Solution: Transfer 4.0 g of the Haemoglobin into a 250-ml beaker, add 100 ml of water, and stir for 10 min to dissolve. Immerse the electrodes of a pH meter in the solution, and adjust the pH to 1.7, stirring continuously, by the addition of 0.3 N hydrochloric acid. After 10 min, adjust the pH to 4.7 by the addition of 0.5 M sodium acetate. Transfer the solution into a 200-ml volumetric flask, dilute to volume with water, and mix. This solution is stable for about 5 days when refrigerated.

Trichloroacetic Acid Solution: Dissolve 14.0 g of trichloroacetic acid in about 75 ml of water. Transfer the solution to a 100-ml volumetric flask, dilute to volume with water, and mix thoroughly.

Sample Preparation

Dissolve an amount of the sample in the Acetate Buffer Solution to produce a solution containing, in each ml, between 9 and 22 HUT. (Such a concentration will produce an absorbance reading, in the procedure below, within the preferred range of 0.2 to 0.5.)

Procedure

Pipet 10.0 ml of the Substrate Solution into each of a series of 25 x 150-mm test tubes: one for each enzyme test and one for the substrate blank. Heat the tubes in a water bath at 40° for about 5 min. To each tube except the substrate blank add 2.0 ml of the Sample Preparation, and begin timing the reaction at the moment the solution is added; add 2.0 ml of the Acetate Buffer Solution to the substrate blank tube. Close the tubes with No. 4 rubber stoppers, and tap each tube gently for 30 sec against the palm of the hand to mix. Heat each tube in a water bath at 40° for exactly 30 min, and then pipet rapidly 10.0 ml of the Trichloroacetic Acid Solution into each tube. (**Caution:** *Do not use mouth suction on the pipet*.) Shake each tube vigorously against the stopper for about 40 sec, and then allow to cool to room temperature for 1 h, shaking each tube against the stopper at 10 to 12 min intervals during this period. Prepare enzyme blanks as follows: heat, in separate tubes, 10.0 ml of the Trichloroacetic Acid Solution in 10.0 ml of the Substrate Solution, shake well for 40 sec, and to this mixture add 2.0 ml of the preheated Sample Preparation. Shake again, and cool at room temperature for 1 h, shaking at 10 to 12 min intervals.

At the end of 1 h, shake each tube vigorously, and filter through 11-cm Whatman No. 42, or equivalent, filter paper, re-filtering the first half of the filtrate through the same paper. Determine the absorbance of each filtrate in a 1-cm cell, at 275 nm, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument to zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained as A_U.

Note: *If a corrected absorbance reading between 0.2 and 0.5 is not obtained, repeat the test using more or less of the enzyme preparation as necessary.*

Standard Curve

Transfer 100.0 mg of L-tyrosine, chromatographic-grade or equivalent (Aldrich Chemical Co.), previously dried to constant weight, to a 1,000-ml volumetric flask. Dissolve in 60 ml of 0.1 N hydrochloric acid. When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 100 µg of tyrosine in 1.0 ml. Prepare three more dilutions from this stock solution to contain 75.0, 50.0, and 25.0 µg of tyrosine per ml. Determine the absorbance of the four solutions at 275 nm in a 1-cm cell on a suitable spectrophotometer versus 0.006 N hydrochloric acid. Prepare a plot of absorbance versus tyrosine concentration. Determine the slope of the curve in terms of absorbance per µg of tyrosine. Multiply this value by 1.10, and record it as A_s. A value of approximately 0.0084 should be obtained.

Calculation

One HUT unit of proteolytic (protease) activity is defined as that amount of enzyme that produces, in 1 min under the specified conditions, a hydrolysate whose absorbance at 275 nm is the same as that of a solution containing 1.10 µg per ml of tyrosine in 0.006 N hydrochloric acid.

Calculate the HUT per g of the original enzyme preparation by the formula,

$$HUT/g = (A_U/A_s) \times (22/30W),$$

in which

22 is the final volume of the test solution,
30 is the reaction time in min, and
W is the weight of the original sample taken, in g.

Note: *The value for A_s, under carefully controlled and standardized conditions, is 0.0084. This value may be used for routine work in lieu of the value obtained from the standard curve, but the exact value calculated from the standard curve should be used for more accurate results and in cases of doubt.*

Proteolytic Activity, Fungal (SAP)

Scope

This procedure is for the determination of proteolytic activity, expressed in spectrophotometric acid protease units (SAPU), of preparations derived from *Aspergillus niger*, var., and *Aspergillus oryzae*, var.

Principle

The test is based on a 30-min enzymatic hydrolysis of a Hammarsten Casein Substrate at pH 3.0 and 37°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized casein in the filtrate is determined spectrophotometrically.

Reagents and Solutions

Casein: Use Hammarsten-grade casein, available from Nutritional Biochemical Corp., 21010 Miles Avenue, Cleveland, Ohio 44128.

Glycine-Hydrochloric Acid Buffer (0.05 M): Dissolve 3.75 g of glycine in about 800 ml of water. Add 1 N hydrochloric acid until the solution is pH 3.0, determined with a pH meter. Quantitatively transfer the solution to a 1000-ml volumetric flask, dilute to volume with water, and mix.

TCA Solution: Dissolve 18.0 g of trichloroacetic acid and 11.45 g of anhydrous sodium acetate in about 800 ml of water, and add 21.0 ml of glacial acetic acid. Quantitatively transfer the solution to a 1000-ml volumetric flask, dilute to volume with water, and mix.

Substrate Solution: Pipet 8 ml of 1 N hydrochloric acid into about 500 ml of water, and disperse 7.0 g (moisture-free basis) of Casein into this solution, using continuous agitation. Heat for 30 min in a boiling water bath, stirring occasionally, and cool to room temperature. Dissolve 3.75 g of glycine in the solution, and adjust to pH 3.0 with 0.1 N hydrochloric acid, using a pH meter. Quantitatively transfer the solution to a 1000-ml volumetric flask, dilute to volume with water, and mix.

Sample Preparation

Using Glycine-Hydrochloric Acid Buffer: Prepare a solution of the sample enzyme preparation so that 2 ml of the final dilution will give a corrected absorbance of enzyme incubation filtrate at 275 nm (A, as defined in the Procedure) between 0.200 and 0.500. Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with Glycine-Hydrochloric Acid Buffer. Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute to volume with Glycine-Hydrochloric Acid Buffer, and mix.

Procedure

Pipet 10.0 ml of Substrate Solution into each of a series of 25 x 150 mm test tubes, allowing at least two tubes for each sample, one for each enzyme blank, and one for a substrate blank. Stopper the tubes, and equilibrate them for 15 min in a water bath maintained at 37° ± 0.1°.

At zero time, start the stopwatch, and rapidly pipet 2.0 ml of the Sample Preparation into the equilibrated substrate. Mix by swirling, and replace the tubes in the water bath. (**Note:** *The tubes must be stoppered during incubation)*. Add 2 ml of Glycine-Hydrochloric Acid Buffer (instead of the Sample Preparation) to the substrate blank. After exactly 30 min, add 10 ml of TCA Solution to each enzyme incubation and to the substrate blank to stop the reaction. (**Caution:** *Do not use mouth suction for the TCA Solution*). In the following order, prepare an enzyme blank containing 10 ml of Substrate Solution, 10 ml of TCA Solution, and 2 ml of the Sample Preparation. Heat all tubes in the water bath for 30 min, allowing the precipitated protein to coagulate completely.

At the end of the second heating period, cool the tubes in an ice bath for 5 min, and filter through Whatman No. 42 filter paper, or equivalent. The filtrates must be perfectly clear. Determine the absorbance of each filtrate in a 1-cm cell at 275 nm with a suitable spectrophotometer, against the substrate blank. Correct each absorbance by subtracting the absorbance of the respective enzyme blank.

Standard Curve

Transfer 181.2 mg of L-tyrosine, chromatographic-grade or equivalent (Calbiochem, La Jolla, Calif. 92037), previously dried to constant weight, to a 1,000-ml volumetric flask. Dissolve in 60 ml of 0.1 N hydrochloric acid. When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 1.00 µmol of tyrosine in 1.0 ml. Prepare dilutions from this stock solution to contain 0.10, 0.20, 0.30, 0.40, and 0.50 µmol per ml. Determine the absorbance of each dilution in 1-cm cell at 275 nm, against a water blank. Prepare a plot of absorbance versus µmol of tyrosine per ml. A straight line must be obtained.

Determine the slope and intercept for use in the Calculation below. A value close to 1.38 should be obtained. The slope and intercept may be calculated by the least squares method as follows:

$$\text{Slope} = [n\Sigma(MA) - \Sigma(M)\Sigma(A)] / [n\Sigma(M^2) - (\Sigma M)^2]$$

$$\text{Intercept} = [\Sigma(A)\Sigma(M^2) - \Sigma(M)\Sigma(MA)] / [n\Sigma(M^2) - (\Sigma M)^2]$$

in which n is the number of points on the standard curve, M is the µmol of tyrosine per ml for each point on the standard curve, and A is the absorbance of the sample.

Calculation

One spectrophotometric acid protease unit is that activity that will liberate 1 µmol of tyrosine per min under the conditions specified. The activity is expressed as follows:

$$\text{SAPU/g} = (A - I) \times 22/(S \times 30 \times W),$$

in which

A is the corrected absorbance of the enzyme incubation filtrate;
I is the intercept of the Standard Curve;
22 is the final volume of the incubation mixture, in ml;
S is the slope of Standard Curve;
30 is the incubation time, in min; and
W is the weight, in g, of the enzyme sample contained in the 2.0-ml aliquot of Sample Preparation added to the incubation mixture in the Procedure.

Proteolytic Activity, Plant

Scope

This procedure is designed for the determination of the proteolytic activity of papain, ficin and bromelain.

Principle

The assay is based on a 60 min proteolytic hydrolysis of a casein substrate at pH 6.0 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration; solubilized casein is then measured spectrophotometrically.

Reagents and Solution

Sodium phosphate solution (0.05 M): Transfer 7.1 g of anhydrous dibasic sodium phosphate into a 1000-ml volumetric flask, dissolve in about 500 ml of water, dilute to volume with water, and mix. Add 1 drop of toluene as preservative.

Citric acid solution (0.05 M): Transfer 10.5 g of citric acid monohydrate into a 1,000-ml volumetric flask, dissolve in about 500 ml of water, dilute to volume with water, and mix. Add 1 drop of toluene as preservative.

Phosphate-cysteine-EDTA buffer solution: Dissolve 7.1 g of anhydrous dibasic sodium phosphate in about 800 ml of water, and then dissolve in this solution 14.0 g of disodium EDTA dihydrate and 6.1 g of cysteine hydrochloride monohydrate. Adjust to pH 6.0 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, then transfer into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

Trichloroacetic acid solution: Dissolve 30 g of trichloroacetic acid in 100 ml of water.

Casein substrate solution: Disperse 1 g (moisture-free basis) of Hammarsten casein or equivalent in 50 ml of Sodium Phosphate Solution, and heat for 30 min in a boiling water bath, with occasional shaking. Cool to room temperature, and with rapid and continuous shaking, adjust to pH 6.0 ± 0.1 by the addition of citric acid solution.

Note: *Rapid and continuous agitation during the addition prevents casein precipitation.*

Quantitatively transfer the mixture into a 100-ml volumetric flask, dilute to volume with water, and mix.

Stock standard solution: Transfer 100.0 mg of USP Papain Reference Standard into a 100-ml volumetric flask, dissolve and dilute to volume with Phosphate-Cysteine-EDTA Buffer Solution, and mix.

Diluted standard solutions: Pipet 2, 3, 4, 5, 6 and 7 ml of Stock Standard Solution into a series of 100-ml volumetric flasks, dilute each to volume with Phosphate-Cysteine-EDTA Buffer Solution, and mix by inversion.

Test solution: Prepare a solution from the enzyme preparation so that 2 ml of the final dilution will give an absorbance in the Procedure between 0.2 and 0.5. Weigh the sample accurately, transfer it quantitatively to a glass mortar, and triturate with Phosphate-Cysteine-EDTA Buffer Solution. Transfer the mixture quantitatively into a volumetric flask of appropriate size, dilute to volume with Phosphate-Cysteine-EDTA Buffer Solution, and mix.

Procedure

Pipet 5 ml of Casein Substrate Solution into each of a series of 25 x 150 mm test tubes, allowing three tubes for the enzyme unknown, six for a papain standard curve, and nine for enzyme blanks. Equilibrate the tubes for 15 min in a water bath maintained at 40 ± 0.1°. At zero time, rapidly pipet 2 ml of each of the Diluted Standard Solutions, and 2-ml portions of the Test Solution, into the equilibrated substrate, starting the stopwatch at zero time. Mix each by swirling, stopper and place the tubes back in the water bath. After 60.0 min. add 3 ml of Trichloroacetic Acid Solution to each tube. (**Caution:** *Do not use mouth suction*). Mix each tube immediately by swirling.

Prepare enzyme blanks containing 5.0 ml of Casein Substrate Solution, 3.0 ml of Trichloroacetic Acid Solution, and 2.0 ml of one of the appropriate Diluted Standard Solutions or the Test Solution.

Return all tubes to the water bath, and heat for 30.0 min allowing the precipitated protein to coagulate completely. Filter each mixture through Whatman No. 42, or equivalent, filter paper, discarding the first 3 ml of filtrate. The subsequent filtrate must be perfectly clear. Determine the absorbance of each filtrate in a 1-cm cell at 280 nm with a suitable spectrophotometer, against its respective blank.

Calculation

One papain unit (PU) is defined in this assay as that quantity of enzyme that liberates the equivalent of 1 µg of tyrosine per h, under the conditions of the assay. Prepare a standard curve by plotting the absorbances of filtrates from the Diluted Standard Solutions against the corresponding enzyme concentrations, in mg/ml. By interpolation from the standard curve, obtain the equivalent concentration of the filtrate from the Test Solution. Calculate the activity of the enzyme preparation taken for analysis as follows:

$$PU/mg = (A \times C \times 10)/W$$

in which

A is the activity of USP Papain Reference Standard, in PU per mg,
C is the concentration, in mg per ml, of Reference Standard from the standard curve, equivalent to the enzyme unknown,
10 is the total volume, in ml, of the final incubation mixture, and
W is the weight, in mg, of original enzyme preparation in the 2-ml aliquot of Test Solution added to the incubation mixture.

Pullulanase Activity

Scope

This procedure is designed for the determination of the pullulanase activity. (Pullulan is produced by deep fermentation of food grade hydrolysed starch by *Aureobasidium pullulans.*)

Principle

Pullulanase hydrolyses α 1-6 glycosidic links in branched poly-saccharides and breaks down pullulan to yield maltotriose only. After the reaction is complete, the reducing sugars formed are estimated by the reaction with dinitrosalicylic acid. Thus one unit of Pullulanase is the activity which will produce reducing sugars equivalent to 1 mg of anhydrous maltose after one min, under the conditions of the assay. (*Maltose is used as the standard of comparison, because maltotriose is expensive and not of the highest purity. The method measures the reducing end groups of maltotriose and higher sugars using maltose as a reference.)*

Reagents

Pullulan solution: Add 1 g of standard pullulan to 70 ml of distilled water. Boil for 5 min, cool and add 10 ml of molar acetate buffer pH 5,0 then dilute to 100 ml. Filter if necessary. This solution can be stored up to two weeks in a refrigerator.

3,5-Dinitrosalicylic acid reagent (DNS): Add 1 g of DNS to 16 ml of 10% w/v sodium hydroxide solution. Add 30 g of Rochelle salt (potassium sodium tartrate tetrahydrate) and 50 ml of distilled water and then warm until dissolved. Dilute this solution to 100 ml. It may be kept for 5 days at 5°.

Procedure

Pipet 1 ml of substrate pullulan solution into a 17 x 1.5 cm test tube and place in a water bath at 50° for 5 min. Add 1 ml of enzyme solution and allow reaction to proceed for exactly 10 min. Stop reaction by adding 2 ml of DNS reagent.

Prepare a blank by adding 2 ml of DNS reagent to substrate before the enzyme is added.

Place the two tubes in a boiling water bath for exactly 5 min and then cool rapidly and add 10 ml of distilled water. Mix solutions well by shaking.

Measure the absorbance of the test solution against the blank using 2-cm glass cells at a wavelength of 540 nm.

Standardization

The reducing value measured is compared with that of a standard maltose solution. A standard maltose graph is not necessary as, for accurate results, the absorbance produced in the test should be between 0.2 - 0.5. As 1 mg of maltose will give an absorbance of 0.82, for the purpose of the calculation the definition is adjusted to read "0.4 units of activity will produce 0.4 mg of anhydrous maltose equivalent...". Therefore a standard maltose solution is made so that 1 ml contains 0.4 mg of anhydrous maltose and this solution is used for the test

in place of the 1 ml of enzyme solution. The absorbance is read as before and should be 0.325. This reading is so constant that, if any difference is found, the wavelength calibration on the spectrophotometer should be checked. This is critical since very small errors in the wavelength can have large effects on the absorbance.

Calculation

For an unknown sample several dilutions are made up and tested. A graph of absorbance against enzyme concentration is plotted (see Figure 2) and the concentration of enzyme which will give an absorbance of 0.325 is found. Then, by definition this concentration of enzyme contains 0.4 Pullulanase units. Thus the activity of Pullulanase preparation is found by:

Pullulanase activity/mg = 1,000 / mg of enzyme in test x (0.4 / 10)

Enzyme concentration	mg in test	Absorbance
0.002%	0.02	0.170
0.003%	0.03	0.245
0.004%	0.04	0.325
0.005%	0.05	0.390
0.006%	0.06	0.465
0.008%	0.08	0.595
0.010%	0.10	0.720

From the graph, an absorbance of 0.325 is given by 0.004% w/v enzyme solution. Therefore the activity equals

(1,000 / 0.04) x (0.4 / 10) = 1,000 units per g

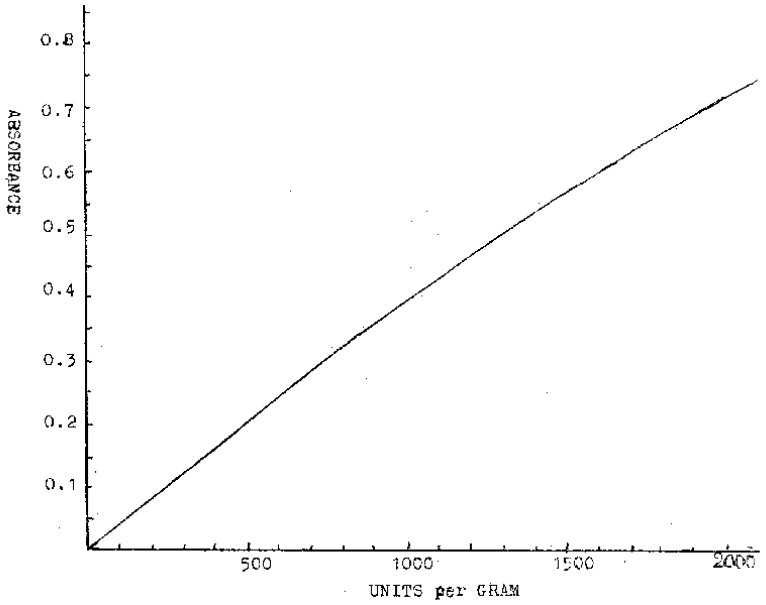


Figure 2. Pullulanase Assay for 50 mg/kg solution

This can now be used to construct a standard graph of absorbance against Pullulanase units for a fixed enzyme concentration. This graph can be used for all further samples. If the 0.005% solution is taken as standard, then its absorbance of 0.39 must give 1,000 units/g (as above). From this, a graph can be constructed for any sample at a concentration of 0.005%

Enzyme concentration	Absorbance	Units/g
0.002%	0.170	400
0.003%	0.245	600
0.004%	0.325	800
0.005%	0.390	1,000
0.006%	0.465	1,200
0.008%	0.595	1,600
0.010%	0.720	2,000

A graph is drawn on absorbance against units/g for a 0.005% enzyme solution.

Example

For an enzyme made up to concentration of 0.0025%, 0.005% and 0.0075%, the absorbances would be:

Concentration	Absorbance	Units g
0.0025%	0,200	480
0.005%	0.375	950
0.0075%	0.553	1,470

Thus the activity is found as follows:

0.0025% 480 x 0.005 / 0.0025 = 960 u/g

0.005% 950 x 0.005 / 0.005 = 950 u/g

0.0075% 1,470 x 0.005 / 0.0075 = 980 u/g

Average = 953 units/g

Xylanase activity (Method 1)

Principle

Xylanase samples are incubated with a remazol-stained wheat arabinoxylan substrate. Unconverted substrate is precipitated with ethanol. The intensity of blue colouring of the supernatant due to unprecipitated remazol-stained substrate degradation products is proportional to the endoxylanase activity. Xylanase activity is measured relative to an enzyme standard and calculated in Farvet Xylanase Units (FXU). The colour profile may vary from enzyme to enzyme.

Apparatus

Spectrophotometer
Thermostatic water bath
Centrifuge
10-ml plastic test tubes
Stopwatch

Reagents and substrates

Phosphate buffer stock solution, 1.0 M: Dissolve 1210 g sodium dihydrogen phosphate monohydrate and 218.9 g disodium hydrogen phosphate dihydrate in demineralised water. Add 40 ml 4 N NaOH and make up to 10 l with water.

Phosphate buffer, 0.1 M, pH 6.00 ± 0.05: Take 1000 ml phosphate buffer stock solution and adjust the pH to 6.0 ± 0.05 using either 4 N NaOH or 2 N HCl. Make up to 10 L with demineralised water.

Azo-wheat arabinoxylan substrate (Megazyme Ltd., Bray, Ireland) 0.5% w/v pH 6.00 ± 0.05: Weigh 0.500 g Azo-wheat arabinoxylan into a 150-ml beaker. Add about 90 ml of 0.1 M phosphate buffer, and heat to approximately 50°, while stirring. Continue stirring at 50° for a further 20 min. Cool the substrate solution and adjust to pH 6.00 ± 0.05 before transferring to a 100-ml graduated flask. Fill to the mark with phosphate buffer.

Stop reagent: Pipette 6.65 ml 2 N HCl into a 100 ml graduated flask. Fill up to the mark with 99.9% ethanol.

Standard solutions: Reference enzyme stock and working solutions: Accurately weigh approximately 1g FXU standard into a suitable graduated flask, add 0.1 M phosphate buffer to volume and dissolve the standard by stirring for approximately 15 min. Use this stock solution to prepare at least 6 FXU standard working solutions to give a range of activities between 0.2 and 1.4 FXU/ml for the construction of the standard curve. Prepare additional samples of known activity for inclusion at the beginning and the end of each analysis series or at least every 20 samples.

Samples: Samples are diluted on the basis of their anticipated activity so that the activity of the final dilution is between. 0.4-1.4 FXU/ml. Results outside the working range may be used to assess the activity of the sample for the next run. Weigh dry or liquid samples directly into the flask. Granulated products may take a considerable time to dissolve.

Procedure

Pipette 0.100 ml working standard or sample solution into 10-ml test tubes, add 0.900 ml of the substrate and mix. Incubate the tubes in a 50° water bath for 30 min. Add 5 ml stop reagent and mix for 10-20 sec.

Leave the tubes to stand at room temperature, for 15-60 min and centrifuge at 4000 rpm for 15 min. Measure the absorbance of the supernatant at 585 nm within 20 min.

Calculation

Use the measurements for the enzyme standards to plot a standard curve. The data may be fitted to a third order polynomial. Determine the corresponding enzyme activity values from the standard curve for the samples. The activity of each sample is calculated as follows:

$$\text{Sample activity (in FXU/g)} = \frac{C \times F \times D}{W}$$

Where:

C is enzyme activity read from the standard curve (FXU/ml)
F is volume of sample (ml)
D is further dilution of sample (e.g. second or third dilution)
W is weight of sample (g)

Xylanase activity (Method 2)

Principle

Xylanase samples are incubated with azurine-crosslinked wheat arabinoxylan substrate. Xylanase hydrolyses the substrate to water-soluble fragments with the concomitant change in colour. The reaction is terminated after a designated time and the optical density (OD) of the reaction mixture is measured at 590 nm (OD_{590}). Xylanase activity is calculated based on the rate of release of the azurine dye. One xylanase unit (XU) is defined as the amount of enzyme that increases the OD_{590} at a rate of one OD per 10 minutes under standard conditions (pH 5.00; 40^{o}).

Apparatus

Spectrophotometer
Magnetic stirrer
Thermostatic water bath
Whatman No. 1 filter paper
Test tubes (15 ml)

Reagents

Citric acid monohydrate
Disodium hydrogen phosphate dihydrate
TRIS (tris (hydroxyl methyl) amino methane)
Sodium hydroxide
Substrate (azurine-crosslinked wheat arabinoxylan: Xylazyme tablets from Megazyme, Ireland)

Note: *a new batch of the substrate should be compared with a previous batch by analyzing the same enzyme preparation using both substrates. If a difference in enzymatic activity is noted, an appropriate correction factor should be calculated and applied to the results obtained with the new batch of the substrate.*

Reaction buffer (McIlvaine buffer, pH 5.00): Dissolve 10.19 g of citric acid monohydrate and 18.33 g disodium hydrogen phosphate dihydrate in 850 ml distilled water in a 1000-ml volumetric flask. Adjust the pH to 5.00 using either 0.1 M citric acid monohydrate or 0.2 M disodium hydrogen phosphate dihydrate. Add water to 1000 ml. The buffer can be stored for up to 6 months at $2\text{-}5^{o}$.

Stop solution (2% w/v TRIS, pH 12.0): Dissolve 20 g of TRIS in 850 ml distilled water in a 1000-ml volumetric flask. Adjust the pH to 12.0 with 5 M NaOH. The solution can be stored for up to six months at $2\text{-}5^{o}$.

Test sample solutions: Accurately weigh a quantity of the enzyme preparation that would give an OD increase within the range of 0.3 – 1.2 in a 100 ml volumetric flask. Add 60 ml of the reaction buffer. Stir the solution using a magnetic stirrer for 10 minutes. Remove the magnet and add the reaction buffer to volume. Transfer the enzyme solution to a glass beaker and let it stand for 5 minutes or until the precipitate settles. Use clear solution for analysis.

Blank: Pre-heat 1.0 ml reaction buffer at 40.0^{o} for 5 min. Add one Xylazyme tablet. After exactly 10 min at 40.0^{o}, add 10.0 ml stop solution and filter the sample through Whatman No.1 filter.

Procedure

Prepare 3 test tubes for each test sample. Pipette 1 ml of the reaction buffer to each tube and add 50, 75, and 100 microliters of the test sample solution.

- Pre-heat all test sample solutions at 40.0^{o} for 5 min.
- Add one Xylazyme tablet to each tube. Do not stir.
- After 10 minutes (±1 sec), terminate the reaction by adding 10 ml stop solution.
- Filter all solutions through Whatman No. 1 filter paper.
- Measure OD of each test sample solution against the blank at 590 nm.

Calculations

Perform linear regression on OD_{590} as a function of test sample volumes (in ml) used in the analysis. Calculate the activity of the enzyme preparation in xylanase units (XU) per gram (g) using the following equation:

$$\frac{XU}{g} = S\frac{V}{W}$$

Where:

S is the slope obtained from linear regression of the OD_{590} as a function of sample volume in ml
V is the volume of the volumetric flask used to prepare the test sample solution in ml (multiplied by further dilutions, if applicable)
W is the weight of the enzyme preparation in g