14 ISSN 1810-1119

FAO ANIMAL PRODUCTION AND HEALTH



manual

QUALITY ASSURANCE FOR ANIMAL FEED ANALYSIS LABORATORIES

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QUALITY ASSURANCE FOR ANIMAL FEED ANALYSIS LABORATORIES

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
Rome, 2011

Information for users of this Manual

Should you face any problem in using methods described in this manual or have a query regarding a method, you may contact experts listed in the FAO Network of Experts: http://www.fao.org/ag/againfo/home/documents/Network_Quality-control.pdf

Recommended citation

FAO. 2011. *Quality assurance for animal feed analysis laboratories.* FAO Animal Production and Health Manual No. 14. Rome.

ISBN 978-92-5-107050-5

Contents

Foreword

Animal feeding impacts on many areas of agriculture: productivity, environmental emissions, water pollution, land use, animal health, product safety, product quality and animal welfare.

Every sector of the livestock industry, the associated services and the wellbeing of both animals and humans are influenced by animal feeding. Proper animal feeding is the supply of a diet balanced in all nutrients and free from deleterious components, at a level that meets the production objective, considering the animal's physiological state, and generates animal products that are safe for human consumption. The availability of accurate, reliable and reproducible analytical data is imperative for proper feed formulation. Also only reliable data can lead to the generation of sound scientific data.

Reports received from international experts visiting animal nutrition laboratories, engaged in analysing feeds and feed ingredients in developing countries, highlight the need to strengthen quality assurance systems in these laboratories. As suitable quality assurance systems are not in place, the laboratory personnel are unable to evaluate the quality of the data being generated. Various ring tests conducted in developed countries have shown an unacceptable variation for some analytes being routinely determined in feed analysis laboratories. Similarly evidence received from the feed industries in developing countries on the reliability of feed analysis data suggests this is inconsistent, therefore, an urgent need to produce a document covering quality assurance systems was realised.

The current document has been developed and prepared by a panel of nine experts. The emphasis is on the basic analysis used for determining the nutritional value of feeds and feed ingredients. The document gives a comprehensive account of good laboratory practices, quality assurance procedures and examples of standard operating procedures as used in individual specialist laboratories. The adoption of these practices and procedures will assist laboratories in acquiring the recognition of competence required for certification or accreditation and will also enhance the quality of the data reported by feed analysis laboratories. In addition, ensuring good laboratory practices presented in the document will enhance the safety of the laboratory workers, protect the environment from laboratory-discharged pollutants and increase the efficiency of laboratories. The document will also provide a strong base for laboratories on which they can develop a system which will meet the requirements of international standards. It will be useful for Laboratory Analysts, Laboratory Managers, research students and teachers and it is hoped that it will enable workers in animal industry, including the aquaculture industry, to appreciate the importance of proven reliable data and the associated quality assurance approaches. This document, through increasing skills and knowledge of laboratory personnel and researchers, will also result in quality assurance systems becoming an integral part of the functioning of a feed analysis laboratory. It will assist countries to initiate the process of getting their feed analysis laboratories accredited to international standards.

An additional effect of implementing and adopting these quality control/assurance approaches will be strengthening of the research and education capabilities of students graduating from R&D institutions and promotion of a better trading environment between

developing and developed economies. This will have long-term benefits and will promote investment in both feed industries and R&D institutions.

This document will also serve as a basis for developing a self-learning e-module and for organising training workshops aimed at Laboratory Managers and Technical Analysts on quality control/assurance approaches. Based on the feedback from users, the document will be expanded in future by including important techniques for feed additives, microbiology, drug residues and other undesirables.

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Acknowledgements

We thank Dr. Jim Balthrop for preparing the initial draft. Special thanks are also due to Ms. Felicity Jackson and Mr. Leon de Jonge for their untiring efforts to collate the information from other contributors and to bring the contents to a uniform format. We are also grateful to Prof. Tim Herrman for his suggestions during the initial phase of the manual preparation. Excellent support provided by Mr. Simon Mack, former Chief of Livestock Production Systems Branch (AGAS); Philippe Anker, Current Chief of AGAS and Samuel Jutzi, former Director of Animal Production and Health Division is highly appreciated.

Glossary of Terms

Accuracy. The difference between an observed or measured value and the accepted or "true value". Since accuracy is affected by both random and systematic errors, accuracy can also be defined as the sum of systematic plus random error.

Blank. A sample containing no added analyte or a sample treated in such a manner that the desired reaction does not take place, e.g. one of the reagents used to produce a reaction is omitted.

Coefficient of Variation (Relative Standard Deviation). The standard deviation divided by the mean and multiplied by 100.

Control Chart. A graphical method for recording measured values which, by using control limits, helps determine whether a process is steady and 'on target'. All control charts have three basic components:

A centreline, usually the mathematical average of all the samples plotted. An upper and lower statistical control limit that define the constraints of common cause variations, and the performance data plotted over time. The use of statistical process control charts allows for the monitoring of variation in laboratory analyses, over a specified time period.

Document. A controlled written policy, procedure, or work instruction that defines what people do and how to do it. Controlled means that the document states who wrote or authorised the policy or procedure, when it was issued and states any version number to avoid the use of a document that is no longer valid. Control of documentation will normally be the responsibility of the Quality Assurance Manager.

Limit of Detection (LOD). The lowest perceivable signal above the background for a particular procedure. The LOD is defined as the mean of the blank plus three standard deviations of the mean of the blank.

Limit of Quantification (LOQ). The lowest experimentally measurable signal obtained for the actual analyte using a particular procedure. The LOQ is defined as the average mean of the blank plus ten standard deviations of the mean of the blank.

Linearity. Where a series of points show less than 3% deviation from a straight line.

Outlier. An observation which deviates so much from other observations as to arouse suspicions that it was generated by a different or erroneous procedure.

Precision. A measure of the scatter of the data around the average.

Proficiency Sample (External Quality Assurance Sample). Samples provided by external source in order to compare laboratory results between laboratories and may be used as an internal quality control sample.

Quality Assurance. Planned and systematic activities implemented within the laboratory that provide confidence in the accuracy and reliability of results generated.

Quality Control. Activities used to monitor a process or to check a result and provide assurance that all activities are performing within pre-determined limits set by the laboratory.

Records. Can be electronic or paper. Examples include chain of custody paperwork, sample results, QA/QC data, audit results, calibration records, etc.

Standard Operating Procedure (SOP). Document describing specified steps taken in a method. This method can be a specific analytical procedure or a policy controlling a more generic aspect of the work performed (e.g. training records, handling complaints or using balances).

Traceability. The property of the result of a measurement whereby it can be related to stated references usually international standards through an unbroken chain of comparisons.

Working control. An internal quality control sample, of known value, analysed with each group of samples in order to monitor the performance of the method and analyst (see Table 2).

Introduction

Availability of animal feed and efficient feeding are the foundations of successful livestock production. The feeding of a balanced ration and correct feed formulation increases animal productivity, quality of product and animal welfare. Also to decrease livestock associated pollution of the environment, feeding of a diet that matches the physiological status of the animal is essential.

For the best health protection of both the animal and human population and to facilitate trade between developing and undeveloped countries, the harmonising of Quality Assurance approaches is imperative.

A robust Quality Management System provides the mechanism to ensure that all these criteria are met and provides a system to constantly monitor laboratory results and identify opportunities for improvement.

A Quality Management System provides management, staff and customers with confidence that all technical, administrative and human factors that influence the quality of the results being generated are under continuous supervision with the aim to prevent non conformity and identify opportunities for improvement.

This manual has been prepared to describe a Quality Management System that may be used by animal nutrition/feed analysis laboratories and serve as a reference source which specific laboratories can use to implement protocols appropriate to their specific situations. However the principles laid down are generalised and may not apply to every laboratory situation.

The Quality Management System described in this manual is based on ISO 17025:2005 principles and is intended to help laboratory personnel maintain the standards expected while providing a consistent, reliable, efficient and professional service with the level of quality required by the laboratory's customers. This policy is achieved through the commitment of management and staff at all levels to apply laboratory practices that ensure the quality of testing services and of the results produced.

Since the work in individual laboratories varies greatly it is essential to have a flexible yet detailed Quality Management System. The laboratory personnel must have an understanding of the principles underlying quality assurance and must apply them in all areas of their work. Only in this way can they maintain credibility, which is the most important attribute of any laboratory. This manual provides a strong foundation for laboratories on which they can develop a Quality Management System which will meet the requirements of the international standard.

The manual has been divided in two main sections. Part I presents general aspects of quality assurance procedures and good laboratory practices that must be put in place in the feed analysis laboratory. Part II contains some basic procedures for determination of nutrients and mycotoxins. The methods described for various analytes have been taken from official recognised methods as well as from the laboratories whose representatives

contributed to the production of this document. The analysts in these laboratories have been using these methods for many years and the methods have proved reliable. However, other methods or variants of the methods presented in this manual may also be used.

It is planned to include a number of important techniques for feed additives, microbiological agents, drug residues and other undesirables and associated quality assurance approaches in the next edition of the manual.

PART I

Quality assurance procedures and good laboratory practices

Setting up a quality laboratory system

QUALITY AND QUALITY CONTROL SYSTEMS

This section explains what is meant by Quality and Quality Management System and how this will impact on and improve working in a laboratory.

What does Quality mean?

Quality is not easy to define but it needs to be defined by an accredited laboratory. It can mean different things to different people. However, in all aspects of business 'Quality' has become very important. Whether it is a manufacturing or a multi-disciplinary organisation, all successful organisations want to be associated with the word 'Quality'.

So what is Quality? A textbook definition is: *Quality is fitness for purpose.*

The International Standards Organisation (ISO) has produced a document entitled 'Quality Management Systems – Fundamentals and Vocabulary', in which the word 'Quality' is defined as: *Degree to which a set of inherent characteristics fulfils requirements.*

This clearly indicates that achieving quality means fulfilling requirements. The requirements may come from customers and in some cases from regulatory authorities.

How can Quality be achieved?

Quality is everyone's responsibility; it must be built in at every stage of the process, from identifying the customer's needs, through planning and implementation right up to the point of reporting analytical results.

In some cases, quality needs to be checked even beyond delivery to the customer since customer satisfaction can have an enormous impact on quality as perceived by them.

Making it happen!

It must be understood that quality does not occur by accident. The starting point is identifying the customer's needs and from that a plan must be considered for the processes and resources and application of monitoring controls. The analyst needs to be continually assessing his/her performance against his/her own objectives and standards to strive for improvement. Since quality does not occur by accident there is a need to establish an effective Quality Management System in order to ensure that requirements are fulfilled efficiently and effectively. This manual is a start to achieving this.

Quality Management System

A Quality Management System directs and controls an organisation with regard to quality by putting in place standard operating procedures (SOPs) to which everyone operates in a

consistent manner. This, in combination with regular internal checks (audits), a system of investigating problems (anomalies) and constantly identifying opportunities for improvement will reduce the occurrence of unreliable results.

Why implement a Quality Management System?

Having a documented system results in all staff operating to a common standard and provides assurance to customers of test reliability and consistency of service. A Quality Management System that complies with an international standard will be recognised around the world and will demonstrate conformity in international markets.

What a laboratory analyst is expected to do to comply?

On commencing employment, all new staff should be issued with a copy of this manual. Staff are expected to read it and understand its contents (where appropriate). Staff should also be issued with a Training File in which they can demonstrate their experience and competency in following the standard, the Quality Management System and their proficiency in nutritional analysis procedures.

Training File

The training file should demonstrate experience and competency to perform the job the individual holds. This file should be held by the individual and updated as training is provided, in liaison with their supervisor. The Training File should contain a job description, an organisational chart, a CV (resume) and evidence of relevant training and education received to date. As new training is received, evidence verified by a line manager, should be included describing the training. Ongoing competency should also be demonstrated by documenting participation in EQA (External Quality Assurance) and IQA (Internal Quality Assurance) programmes managed by the laboratory.

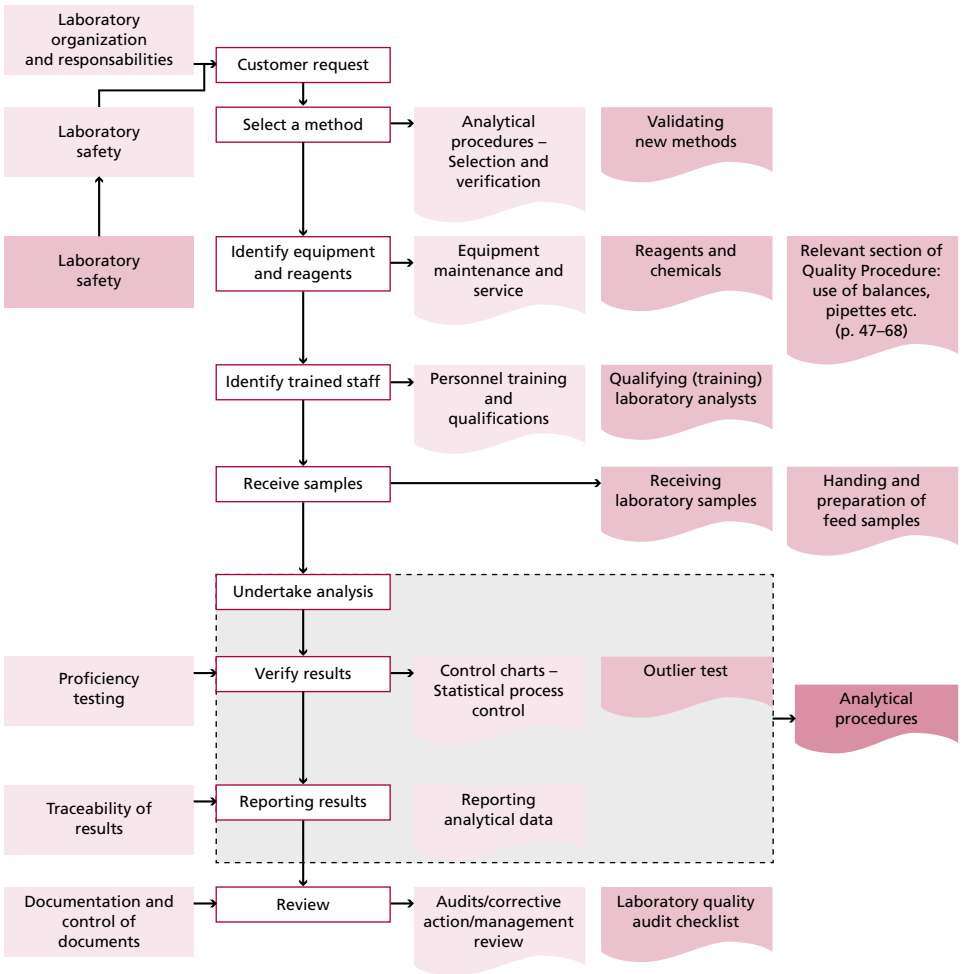
QUALITY ASSURANCE PURPOSE AND GUIDELINES

Laboratory quality programs are a critical part of improving the agricultural laboratories in developing countries. The Laboratory Quality Manual is the essential source for communicating to the laboratory staff the manner in which laboratory testing is to be conducted. Adherence to the quality manual by laboratory staff is necessary to ensure both quality and consistency. Recognizing the Laboratory Manual may not cover all situations and variables arising from the laboratory setting, any significant departures must have the approval of management and must be appropriately documented.

The management within the laboratory is responsible for the quality and integrity of all data generated in the laboratory. The management, collectively, assures this quality through adherence to the laboratory manual, quality assurance plan, and through the development and adherence to standard operating procedures.

The flowchart in Figure 1 provides a simplified representation of the Quality Management System described in this manual and is not a substitute for the procedures contained within.

FIGURE 1
Quality Assurance flowchart



Note: Light coloured boxes refer to Part I – Setting up a quality laboratory system; darker coloured boxes refer to Part I – Quality procedures; darkest coloured boxes refer to Part II.

LABORATORY ORGANIZATION AND RESPONSIBILITIES

Each member of the laboratory should have clearly identified and documented responsibilities (Job Description). An organizational chart should be included in the laboratory quality document and made available in staff training records.

Laboratory Manager/Director has ultimate responsibility for implementing the quality system.

Quality Manager reports directly to the Laboratory Manager/Director and is responsible for maintaining and developing the quality procedures used in the laboratory.

Laboratory analysts responsible for following all quality procedures and identifying opportunities for improvement.

PERSONNEL TRAINING AND QUALIFICATION

Qualified and trained personnel are essential for producing analytical results of acceptable quality. Laboratory management ensures that laboratory personnel have the knowledge, skills and abilities to perform their duties. Competence is based on education, experience, demonstrated skills and training. Staff training files contain the documentation of personal education, experience, skills and training for the position held.

Analysts undergo a training program in accordance with the laboratory's training procedure. The analyst must demonstrate and document proficiency in an analytical method before reporting results to the laboratory's customers. The first step for qualifying in a new analysis is to read the standard operating procedure (SOP). A copy of this document can be obtained from the Laboratory Manager/Director. The method should be reviewed with the analyst by someone familiar with the procedure and then the analyst should run a specified number of known samples or standards. The training should be documented in the individuals training file. To assure the safety of everyone, the trainee must read the Material Safety Data Sheet (MSDS) for information concerning each chemical used in the analysis. The toxicity levels and method of waste disposal should be clearly understood before beginning any analysis. The number of samples and standards analysed should be specified by the supervisor. The results should be compared to previously obtained results using a paired t-test. If there is no significant difference at the 95% confidence level the new analyst can be considered qualified. On-going competency should be demonstrated by participation in Internal Quality Assurance (IQA) or ring trials at regular intervals.

ANALYTICAL PROCEDURES – SELECTION AND VERIFICATION

When the customer does not specify the method to be used, a recognised (ISO, CEN, AOAC, FDA etc.) standard method is preferred. If a standard method is not found the laboratory may use either a non-standard method or modify a method for use with the concurrence of the customer. The laboratory informs the customer when the method proposed by the customer is considered to be inappropriate for the intended purpose. The standard and non-standard or modified method must be sufficiently validated by the laboratory before being used to report data.

When the laboratory develops methods for its own use, the laboratory has a procedure for its introduction.

Non-standard methods are those methods not taken from authoritative, validated sources. A non-standard method has not undergone validation, such as a collaborative study or process to evaluate the method's performance capabilities.

Non-standard methods are selected for use when a customer request cannot be addressed with the use of a standard method. Such methods are subject to agreement with the customer and are validated.

Validation is the confirmation by examination and the provision of objective evidence that the particular specifications for an intended use are fulfilled.

The laboratory validates standard methods, non-standard methods, laboratory developed methods and modified standard methods, including use outside the intended scope and applications. Validation is conducted to confirm that the methods are fit for the intended use. The performance of all methods is verified before being used to generate reportable data.

The validation process addresses the needs of the given application. The attributes and data quality objectives include but are not limited to:

- Accuracy
- Precision
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Linearity

Accuracy and precision target limits can be taken from AOAC (Horwitz). Precision or repeatability is calculated as the relative standard deviation (coefficient of variability) and accuracy is calculated as percentage recovery (Table 1).

STANDARD OPERATING PROCEDURES (SOPs)

SOPs are specific to the point of use for which they are written. The approval of a SOP is the commitment of a specific area to an action or behaviour. SOPs may be written by a competent employee within the laboratory. The SOP is then reviewed for content and authorised by the supervisor or manager in the area in which the SOP will be used. Once the SOP has been reviewed and found to be acceptable by the supervisor or manager, the SOP is given to the Quality Manager for approval and issue.

The format used for writing SOPs should contain the following when appropriate:

- Principle
- Scope
- Responsibilities
- Equipment
- Reagents
- Procedure
- Quality Control
- Calculations
- Troubleshooting
- Remarks
- References
- Appendix (Flow Charts, Tables, References, etc.)

SOPs are controlled documents and must include an issue (or effective) date, the name of the author(s) and person(s) authorising the SOP, a review date and version number. When a new version of an SOP is issued a 'Log of Updates' will summarize changes made at the start of the document. When a new version of an SOP is issued all previous controlled versions must be withdrawn.

It is advisable to forbid uncontrolled versions of SOPs.

EQUIPMENT MAINTENANCE AND SERVICE

The procedures to determine, maintain, and monitor instrument performance are an integral part of a quality control program as they promote a high degree of confidence in analytical results. Each procedure is described in the respective equipment SOP. All essential testing equipment has its own SOP and maintenance records that document operation, calibration and routine maintenance. Staff using essential equipment must have appropriate training documented in their training file.

Maintenance. Complete and accurate installation instructions, operating manuals, parts manuals, service manuals and written guarantees or contracts are kept with each instrument to assure proper functioning. The implementation of a preventive maintenance program which includes the testing of equipment against specifications and procedures for frequent calibration, checking and cleaning, is essential. The performance of instruments and equipment is evaluated on a regular basis to ensure that the equipment or instrument continues to function properly and has the appropriate historical records to properly audit and to evaluate. Routine maintenance tasks such as cleaning, adjusting, replacing of parts or lubricating are performed on each instrument by the responsible officer according to instructions provided in the operating manual of the instrument or as identified by past experience.

All maintenance tasks and repairs performed by the analyst or the service representative are recorded on the current instrument log sheet for each instrument. Analysts must report all malfunctions immediately to the responsible person for the instrument and clearly indicate that it is "OUT OF ORDER" when a malfunction occurs.

When available, service contracts which include semi-annual/annual maintenance of certain pieces of equipment should be obtained.

Calibration. The validity of the analytical results produced is strongly related to the performance level of the instrumentation used for analysis. It is therefore essential that for each instrument and each method, proper calibration procedures be established and that calibration results be recorded and used as a basis for continuous assessment of the instrument performance. The method calibration requirements are included as part of the method. Equipment/instrument calibration and traceability to national standards should be documented. Certified weights and thermometers are available and should be used and the documentation maintained in the laboratory.

Inventory. A permanent inventory record of all equipment is kept by the Laboratory Manager/Director. This record includes the equipment's name, model number, serial number, manufacturer, date of acquisition, original cost and present location and any unique identifier assigned locally.

Parts and Supplies. The analyst must maintain a list of spare parts kept that are critical to keep the instrument operating and must review this list at least annually. This list is to be kept in the Instrument Log Book.

Responsible Officer. A responsible officer is assigned to the essential equipment. This person is normally the principal user of the system. In the case of shared equipment the responsible officer carries out performance checks and maintenance while the operators verify the calibration, run standards, operate the instrument in the correct manner, record data as required and informs the responsible officer of any anomalies or malfunctions.

The duties of the responsible officer are as follows:

1) To become thoroughly familiar, through training and experience, with the operation, maintenance and applications of the instrument.
2) To instruct and assist others in the use of the instrument.
3) To carry out regular performance checks on the instrument as outlined in the instrument and laboratory manuals.
4) To perform routine maintenance tasks according to the instrument manual.
5) To ensure the instrument log sheet or log book is filled out with each use.
6) To ensure that the instrument manuals are readily available and updated as required.
7) To establish a list of spare parts critical to keep the instrument operating and to ensure that there is an adequate supply of these parts. Alternatively a service and maintenance contract may be set up with a suitable sub-contractor.

A nominated deputy should be identified to perform these tasks in the absence of the Responsible Officer.

Instrument Log Book. The main purpose of the instrument log book is to provide a permanent record of instrument performance and to be used as a basis for validating data and projecting repair and replacement needs, or new acquisitions. If applicable, the service contract number is recorded in the log book.

Each time the instrument is used, the analyst must enter the information requested in the Log Book which will provide the laboratory with a record of use of the instrument, its performance and also any maintenance and repairs.

REPORTING ANALYTICAL DATA

Each procedure must specify the applicable range of the analyte to which it may be applied with some realistic significance (test limitation). The Laboratory Analyst is obligated to report numerical values which include only those numbers that are certain plus one digit that is estimated. This group of numbers is referred to as a significant figure. If more than one uncertain number is reported, a reader might be misled concerning the precision to which a measurement or set of measurements was made. To standardise the reporting of laboratory data, two conventions should be used. One deals with the rounding of numbers and the other with the reporting of significant figures. When it is necessary to round off data, round the number to the next higher value if the digit to its right is ≥ 5. If the last number discarded is < 5, leave the last retained digit unchanged. If the number to the right is 5, followed by zeroes only, round to the nearest even number.

The definition offered for significant figures is that they include all numbers in a result known with certainty plus one uncertain value. The position of the decimal point is

irrelevant. When data are reported, use the following rules to determine the number of significant figures:

1) Report only as many significant figures as are found in the least accurate measurement; and
2) Give the reader the best estimation of the errors in the measurement.

Some examples: If the zero is bounded on both the left and right by another number, it is always significant, 306 has three significant figures. If the zero is used to fix the decimal point it is never significant, 0.0024 has two significant figures, 0.00240 has three significant figures.

ACCURACY AND REFERENCE SAMPLES

To assure the accuracy of the procedure a reference sample (working standard) with known and stable values should be run with each batch and evaluated by means of a control chart (see section on control charts).

The reference material can be a pure substance Reference Material (RM) and the recovery of the analyte will be a measure of the accuracy of the method.

For most feed analyses however, Certified Reference Material (CRM) or a home-made feed reference sample (HRM) is used.

The CRM can be obtained from organisations for proficiency tests of animal feeds (Table 4) where the reference values are determined by several laboratories applying several independent validated test methods. A lab can also make its own reference sample. A feed material should be chosen which is representative for the bulk of feed samples analysed in the lab. This sample should be analysed in duplicate, at least in 6 different runs, spread over several days/weeks. In these runs preferably a CRM is analysed and only when the values of the CRM are within the control limits, the results of the HRM may be taken into account. Before calculating the mean and SD clearly deviating results should be eliminated. From the HRM a sufficient amount should be portioned; a portion should be sufficient for at least six months and the remaining portions should be preserved in the freezer. The control chart should be regularly evaluated for trends, which may be indicative of the deterioration of the quality of the portion. The same HRM may be used for several methods in the lab.

PRECISION AND BLIND DOUBLE SAMPLES

To increase the precision of the results (reduce the scatter), all analyses are preferably carried out in duplicate. As this is not always feasible because of financial reasons or sample amount, it is suggested that a minimum 10% of the samples in a batch be run in duplicate. The duplicated sample(s) should be preferably unknown for the analyst (double blind), so ensuring the precision of the samples analysed in singular. The acceptable range of the duplicate results varies depending on the method, the customer requirements and the sample matrix. The relative range or relative percent difference can be calculated as:

Relative Percent Difference = $(X_1 - X_2) \times 100$ / mean of replicate values

where,

X_1 = the largest replicate value

X_2 = the smallest replicate value

For guidelines to the analytical variation that can be expected from analysing a sample twice (Table 1). The analytical variation in this case is two times the coefficient of variation or relative standard deviation:

TABLE 1
Analytical Variations (AV) in [%]; x = analyte concentration

Analyte	AV (%)
Moisture (Dry Mass)	12
Protein	20/x + 2
Fat	10
Crude Fibre	30/x + 6
Ash	45/x + 3
Total sugars as invert	12
Calcium	10
Phosphorus	3/x + 8
Salt	7/x + 5
Vitamin A	30

Source: From the Association of American Control Officials 2011, Official Publication 2011, page 298-299.

For guidelines regarding the accuracy or percent recovery of quality control samples one can use the AOAC "International Guidelines for Single Laboratory Validation of Chemical Methods" (Table 2).

TABLE 2
Acceptable Recovery Limits

Concentration	Recovery Limits (%)
100%	98 - 101
10%	95 - 102
1%	92 - 105
0.1%	90 - 108
0.01%	85 - 110
10 µg/g (ppm)	80 - 115
1 µg/g (ppm)	75 - 120
10 µg/kg (ppb)	70 - 125

To calculate the percent recovery from a reference material:
Percent recovery % = $(X_r / X_k) \times 100$
where,

X_r = observed value of reference material
X_k = certified or true value of reference material

TRACEABILITY OF RESULTS

Since all measurements made by the laboratory must be traceable to the International System of Units (SI), contracting metrologists should provide evidence of measurement traceability of its own measurement standards and measuring instrument to the SI. Further, they should provide documentation demonstrating measurement capability and competence to perform the calibration services requested by the laboratory. The calibration certificates for laboratory instruments (balances, pipettes, etc.) should include the measurement results along with the measurement uncertainty and a statement of conformance with an identified metrological specification. Purchased standards should be accompanied by certificates of analysis.

PROFICIENCY TESTING (EXTERNAL QUALITY ASSURANCE)

Participating in proficiency sample test programs allows for the assessment of the laboratory's accuracy and precision. Where possible, the program's matrices and tests should match that of the laboratories. In order to evaluate the laboratory's performance a "Z" value is calculated. Any Z value ≤ 2 is satisfactory, values between 2 and 3 are questionable and any value ≥ 3 is unsatisfactory, requiring an investigation and corrective action. The Z value is calculated by taking the difference between the laboratory's result and the program's reported result and dividing that difference by the programs reported standard deviation. In other words, the Z value is how many standard deviations the laboratory's result is from the consensus value. The Association of American Feed Control Officials (AAFCO), the American Association of Cereal Chemists (AACC), the American Oil Chemists' Society (AOCS) all provide such programs. The European PT Information System (EPTIS) maintains a current list of available proficiency programs. The laboratory should have a documented schedule for participation in proficiency schemes (Table 3).

TABLE 3
Organisations providing proficiency testing and reference samples of animal feed

Organisation	Address	Telephone	Email	Website
AAFCO (Association of American Feed Control Officials)	175 S. University Street IN47907-2063 West Lafayette Indiana USA	+1 7654941565	vsiegel@purdue.edu	www.aafco.org
IAG – Feedingstuffs (International Analytical Group, section feedingstuffs)	191 Spargelfeldstrasse 1220 Vienna Austria	+43 5055532700	Renate.oeschlmueller@ages.at	www.ages.at
BIPEA (Bureau InterProfessionnel d'Etude Analytique)	6-14 av. Louis Roche F-92230 Gennevilliers France	+33 147335460	Contact@bipea.org	www.bipea.org
FAPAS (Food Analysis Performance Assessment Scheme)	Sand Hutton YO41 1 LZ York UK	+44 1904462100	info@fapas.com	www.fapas.com
LGC (Laboratory of the Government Chemist)	1 Chamberhall Business Park BL9 0AP Lancashire UK	+44 1617622500	customersservices@lgcpt.com	www.lgc.co.uk
WEPAL (Wageningen Evaluating Programmes for Analytical Laboratories)	P.O. box 8005 NL-6700 EC Wageningen The Netherlands	+31 317482337	Info.wepal@wur.nl	www.wepal.nl

CONTROLS CHARTS – STATISTICAL PROCESS CONTROL

To ensure that the laboratory methods are in statistical control, laboratory performance should be evaluated using control charts. Mean or X-bar charts are designed to point out changes in the value of the long term average for a sample analysed by a specific method. Range, or R-bar, charts are designed to point out changes in the repeatability of a procedure. If a statistics software program is not available, the data may be plotted using the following procedures:

Construct the basic charts by calculating the long term or grand average mean and grand average range by averaging at least 6 sets of duplicates obtained over 3 days. Make the X bar chart by drawing a solid horizontal line across the centre of the page, label it "0"+, draw dashed lines at ± 1.25 (these represent the warning limits at 95% confidence. Draw two more lines at ± 1.88. These represent the control limits at 99% confidence. Label the y-axis with these values and what they represent, i.e. 1.88 is the upper control limit and -1.88 is the lower control limit. Average the two results of the control samples. Calculate and plot the Z values along the Y axis with the corresponding date along the X axis. Z value = (average of two controls minus grand mean) divided by the grand average range. Construct the R-bar chart by a drawing solid horizontal line across the bottom of the page, labelling it "0". Draw dashed lines one at ± 2.51 which represents the warning level at 95% confidence and the second at ± 3.27 which represents the control limit at 99% confidence. Divide the difference of the two control samples by the grand average of the range of the controls and plot this on the chart along the Y axis with the date along the X axis.

Control charts should be evaluated after each group of samples is analysed and checked to ensure they are within specifications before the results are reported to the customer.

The process is in control if:

1) All points on the X bar and R bar charts are within the control limits (subject to limitations listed below).
2) One and only one mean value in the last 20 is outside the control limits and the range value is not.
3) One and only one range value in the last 20 is outside the control limits and the value of the mean is not.
4) Specific cause for an apparent out of control point has been identified and eliminated.

The process is out of control with respect to the mean (X bar chart) if:

1) More than one mean point in the last 20 has exceeded the upper or lower control limits but the range is within control.
2) Both individual values of the mean for a given control are outside the warning limits even if the mean is not.
3) Seven consecutive means are all on one side of the "0" line.
4) Seven consecutive means fall in a consistent upward or downward pattern.
5) There is a run of four means between the upper warning limit and the upper control limit or between the lower warning limit and lower control limit.

The process is out of control with respect to the range (R bar chart) if:

1) More than one point in the last twenty has exceeded the control limit and both values of x are outside the warning limits on the X chart.
2) The points on both the X bar and R bar charts are outside the control limits.
3) Seven consecutive points fall in a consistent upward or downward pattern in the points.
4) There is a run of four points between the warning limit and the control limit.

A method is not out of control if the analyst knows that he/she did something different or some difficulty was experienced with the procedure or equipment on a given set. This should be recorded on the appropriate worksheet. The control charts only allow for determining whether or not the variation observed can be described by random variation. The acceptable variation or limits are determined by the laboratory customer.

See Figure 2 and Figure 3 for example Mean and Range control charts.

DOCUMENTATION AND CONTROL OF DOCUMENTS

Quality documents that form the management system must be controlled. Laboratory document control procedures describe the process for controlling those quality documents required for the generation of laboratory data. These documents include those published by the laboratory and those published externally. Documents of external origin include regulations, standards, test methods, instructions and manuals.

Documents issued to personnel in the laboratory as part of the management system are reviewed and approved for use prior to issue in accordance with the laboratory's document control and management procedure. The laboratory's master list of procedures identifies the current revision status and distribution of documents. Through the use of the master list, quality documents are issued to personnel to preclude the use of obsolete documents.

The laboratory's master list and document control and record management procedure provide for the following:

- Authorised management system documents and external documents are held at point of use.
- Documents are reviewed according to a schedule and revised to ensure continuing suitability and conformance with the management system, an appropriate revision date will be given to each document.
- Invalid or obsolete documents are promptly removed from all points of issue or use to assure against unintended use.
- Obsolete documents retained for either legal or knowledge preservation purposes are marked as archived or obsolete.
- To avoid the potential of different procedures being in use at the same time at different locations hand amendments or uncontrolled copies should not be permitted.
- A document control header, as described in the laboratory's document control and management procedure, uniquely identifies management system documents generated by the laboratory. Such identification includes the date of revision, identification number, issuing authority and pagination.
- Proposed changes to documents are reviewed and approved in accordance with

FIGURE 2
X-Bar chart for Acid Detergent Fibre (UCL, Upper control limit; LCL, lower control limit)

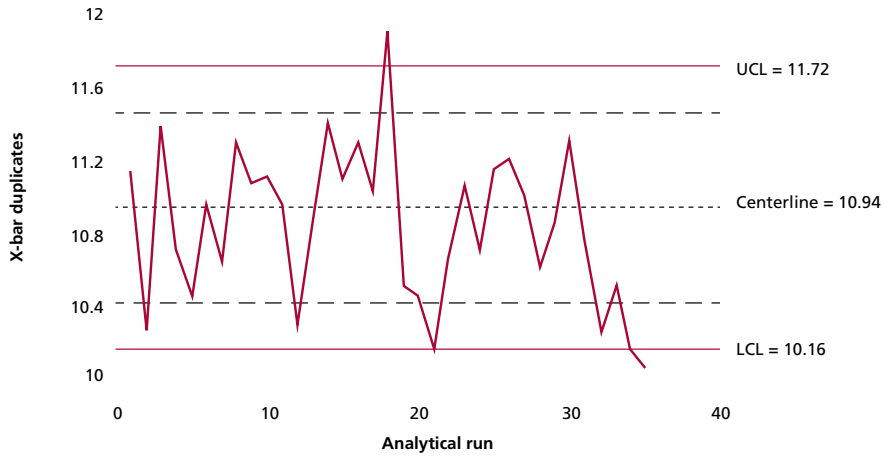
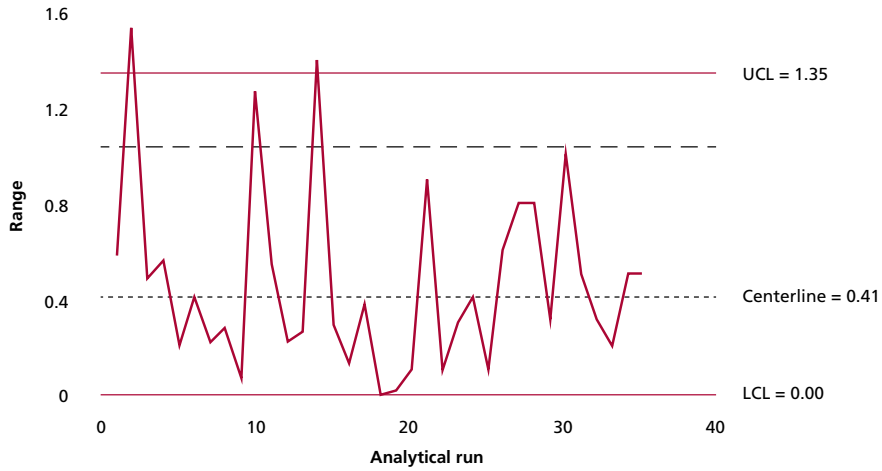


FIGURE 3
Range chart for Acid Detergent Fibre (UCL, Upper control limit; LCL, lower control limit)



the laboratory's document control and management procedure. Unless designated otherwise, this procedure is followed by the same personnel as in the original review or approval.

- The altered or new text is identified either in the document, on a cover page, or in the attachments. A Log of updates, included in the first page of a new document, simplifies identification of any changes.

- The laboratory's document control and management procedure addresses the control of electronic management system documents.

LABORATORY SAFETY

As an employee you have a responsibility to do your job well and safely. A priority of the laboratory is to offer you a workplace free from recognisable and avoidable hazards to your health and safety.

Safety, however, cannot be mandated, nor is it something that can be given to an employee. Rather, you must make a conscious effort to help ensure safe conditions for yourself and for other workers in the area. This requires an understanding of potential hazards on the job and knowledge of the policies and regulations in dealing with those hazards.

There are unavoidable sources of risk in any work environment, particularly in a laboratory. To keep the hazards from causing injuries, each individual must know how to use the tools and equipment safely and be informed of what to do in case of a fire, injury or other emergency. However, information is not enough. Safety on the job is an attitude as much as it is knowledge. It means recognising that accidents are not limited to those people who do not know how to prevent them. It is often the seasoned veteran, the person who "knows better," who becomes a victim by allowing familiarity to dull the edge of caution.

You should maintain constant awareness. It involves your personal commitment to do every job safely.

Other workers must be alerted to danger if they are not following the safety procedures, these incidences **must be documented.**

Supervisors and Safety officers should be identified. These personnel should be notified immediately of defective emergency equipment or other potential dangers.

By participating on safety committees, assisting in making safety inspections and assuring that all safety practices are carried out every time you can ensure all work is done in a safe manner.

No work is so important that there is not time to do it safely and correctly!

For further information on Health & Safety in the laboratory see 'Laboratory Safety' (see page 61).

AUDITS/CORRECTIVE ACTIONS/MANAGEMENT REVIEWS

Internal audits are conducted as needed (minimum of annually). The internal audits are conducted to verify that operations continue to conform to the requirements of the quality management system.

The internal audit schedule addresses all elements of the management system, including analytical activities. The Laboratory Quality Assurance Manager is responsible for the coordination of internal audits in addition to any additional audits requested by management or identified through anomalies.

Trained and qualified personnel are responsible for conducting internal audits. Auditors may audit in their own area but must not audit their own work.

When audit findings cast doubt on the effectiveness of the operations or on the cor-

rectness or validity of the laboratory's analytical results, the laboratory's corrective action procedure is initiated.

The customer is notified if investigations show that non-conformances related to audit results have affected work performed for the customer. This notification is documented and an impact assessment carried out to identify possible anomalous results issued prior to identification of the finding.

The area of activity audited, the audit findings, and corrective and preventive actions (CAPA) that arise from them are recorded according to the laboratory's audit procedure.

Follow-up audit activities are conducted to verify and record implementation and effectiveness of the corrective action taken. This follow-up is included as part of the management review process.

The laboratory's management review procedure includes the schedule for conducting management reviews. This review is conducted by the laboratory's executive management to ensure continuing fitness for use and effectiveness of the management system and to introduce needed changes and identify opportunities for improvements.

The management review addresses the elements of the management system and includes but is not limited to the following elements:

- Suitability of policies and procedures;
- Reports from managerial and supervisory personnel;
- Outcome of recent internal audits;
- Corrective and preventive actions;
- Assessments by external bodies;
- Results of inter-laboratory comparisons (proficiency tests);
- Changes in the volume and type of work;
- Customer feedback;
- Complaints; and
- Other factors, such as quality control activities, resources and staff training.

The findings and the actions that arise from the review are recorded according to the laboratory's management review procedure. Each action includes a target date for resolution.

Quality procedures

VALIDATING NEW METHODS

Summary

This procedure describes how the laboratory should select and validate new laboratory analytical procedures. The laboratory will use methods which meet the needs of its customers. Standard methods are preferable; however, non-standard and laboratory developed methods may be used when deemed more appropriate.

Scope

This procedure applies to all analytical procedures used in the laboratory.

Responsibility

Laboratory Analyst investigates available methods, performs testing, generates and analyses data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Procedures

Selection of method

The need for a new method will be determined. This may be initiated by the Lab Manager, Quality Manager, Laboratory Analyst, or customer.

Available methods will be investigated and evaluated by the Laboratory Analyst and Lab Manager. Cost/benefits will be part of the evaluation process.

The selected method will be approved by the Quality Manager.

Validation/Verification of method

1. Determine method development procedures (Quality Manager).
2. Laboratory Manager/Director will assign a Laboratory Analyst(s) to the project.
3. Laboratory Analyst will procure appropriate chemicals, supplies, and equipment.
4. Laboratory Analyst will prepare written documentation summarising procedures to be used. *NOTE:* Modifications to the procedures used are often required.
5. Quality Manager will approve the procedures.
6. Laboratory Analyst will collect preliminary data and submit the results to the Quality Manager for review.
7. Laboratory Analyst will complete collection of data.
8. Laboratory Manager/Director will review data, prepare summary, and submit report to Quality Manager.
9. A SOP will be prepared.

Minimum Validation Acceptance Criteria

1. **Accuracy/Recovery.** A minimum of 7 independent analyses per concentration level covering the analytical range. It is preferable to use certified reference materials (CRM). If CRMs are not available proficiency test program samples may be used. Purified standards/chemicals can be used if no other reference samples are available. Spiked samples may also be required. The least preferred method to determine accuracy is to compare the results to those results obtained using another already validated procedure.
2. **Precision.** Typically for a single lab validation the Laboratory Analyst should perform *r* replicate analyses of *m* test portions over a period of *d* days for each sample type (matrix) *n*, where *r* is the number of replicates (2, 3,…), *m* is the number of test portions in each group, *d* is the number of days, and *n* is the number of different sample types.
   r x *m* should never be less than 10
   n should be at least 2 (preferably more)
   d should be at least 2
3. **Calibration.** The calibration line should include 4–5 reference points. Single point calibrations must be used cautiously. Correlation coefficients of > 0.99 should be the goal but with modern line fitting software non linear calibration lines are acceptable.
4. **Limits of detection (LOD) and limits of quantification (LOQ).** For some customers LOD must be demonstrated. Typically this is defined as the analyte concentration at 3 times the signal produced by analysing a blank. For many laboratories the LOD is not as significant as the LOQ. Typically this is defined as the analyte concentration at 10 times the signal produced by analysing a blank. However, the useful LOQ is the lowest analyte level the laboratory can measure with an accuracy and precision acceptable to its customer. Unless otherwise specified the laboratory should document the typical LOD/LOQ (3x and 10x blank). *NOTE:* In general laboratories may not report any analytical results that are not bracketed by the calibration curve.
5. **Criteria.** Accuracy and precision target limits may be taken from AOAC (Horwitz). Precision or repeatability is calculated as the relative standard deviation (coefficient of variability) and accuracy is calculated as % recovery (Table 1).

TABLE 1
Accuracy and precision target limits

Concentration	Repeatability (%)	Recovery (%)
100 %	1.3	98 – 102
10 %	1.9	98 – 102
1 %	2.7	97 – 103
0.1 %	3.7	95 – 105
0.01 %	5.3	90 – 107
0.001 %	7.3	80 – 110
1 ppm	11	80 – 110
100 ppb	15	80 – 110
10 ppb	21	60 – 115
1 ppb	30	40 – 120

Validating NIR Calibrations

Principle

The sample representing the chemical composition of the sample material is measured by NIR spectrometry. Spectral data in the near infrared region are collected and transformed to constituent or parameter concentrations by calibration models developed on representative samples.

Near-infrared (NIR) instruments

NIR instruments are based on diffuse reflectance or transmittance measurement in the near infrared wavelength region of 700–2500 nm (14300–4000 cm^{-1}) or segments of this or at selected wavelengths or wave numbers. The optical principle may be dispersive (e.g. grating monochromators) interferometric or nonthermal (e.g. light emitting diodes, laser diodes and lasers). The instrument should be provided with a diagnostic test system for testing photometric noise and reproducibility, wavelength/wave number accuracy and wavelength/wave number precision (for scanning spectrophotometers). The instrument should measure a sufficiently large sample volume or surface to eliminate any significant influence of inhomogeneity derived from chemical composition or physical properties of the test sample. The sample path length (sample thickness) in transmittance measurements should be optimized according to the manufacturer's recommendation with respect to signal intensity for obtaining linearity and maximum signal/noise ratio. In reflectance measurements, a quartz window or other appropriate material to eliminate drying effects should preferably cover the interacting sample surface layer.

Calibration and initial validation

The instrument has to be calibrated before use. This procedure deals with calibrations that have been produced in-house or have been purchased from an external provider.

The calibration must have a sufficient number of representative samples, covering variations such as:

a) Combinations and composition ranges of major and minor sample components;
b) Seasonal, geographic and genetic effects on forages, feed raw material and cereals;
c) Processing techniques and conditions;
d) Storage conditions; and
e) Sample and instrument temperature.

Calibrations must be provided with statistical information on its performance such as number and type of samples, correlation, standard error of calibration and prediction and in a format that allows for the identification of outliers.

Reference analyses and NIR measurements

Internationally accepted reference methods for determination of moisture, fat, protein and other constituents and parameters should be used.

The reference method used for calibration should be in statistical control, i.e. for any sample, the variability should consist of random variations of a reproducible system. It is essential to know the precision of the reference method.

Outliers

In many situations, statistical outliers are observed during calibration and validation. Outliers may be related to NIR data (spectral outliers, hereafter referred to as x-outliers) or errors in reference data or samples with a different relationship between reference data and NIR data (hereafter referred to as y-outliers).

For the purpose of validation, samples are not to be regarded as outliers if they fulfil the following conditions:

a) They are within the working range of the constituents/parameters in the calibration(s);
b) They are within the spectral variation of the calibration samples, as e.g. estimated by Mahalanobis distance; and
c) And if the spectral residual is below a limit defined by the calibration process.

Dealing with outliers. If a sample appears as an outlier then it should be checked initially to see if it is an x-outlier. If it exceeds the x-outlier limits defined for the calibration it should be removed. If it is not an x-outlier, then both the reference value and the NIR predicted value should be checked. If these confirm the original values then the sample should not be deleted and the validation statistics should include this sample. If the repeat values show that either the original reference values or the NIR predicted ones were in error then the new values should be used.

Validation of calibration models. Before use, calibration equations must be validated locally on an independent test set that is representative of the sample population to be analysed. For the determination of bias, at least 10 samples are needed; for the determination of Standard Error of Prediction (SEP) at least 20 samples are needed.

Validation must be carried out for each sample type, constituent/ parameter and temperature.

NOTE: The validation exercise is valid only for the sample types, range and temperature used in the validation.

Results obtained on the independent test set are plotted, referenced against NIR, and residuals against reference results, to give a visual impression of the performance of the calibration. The SEP is calculated and the residual plot of data corrected for mean systematic error (bias) is examined for outliers, i.e. samples with a residual exceeding 3 x SEP.

If the validation process shows that the model cannot produce acceptable statistics then it should not be used.

Validation criteria

The next step is to fit NIR and reference data by linear regression (reference = a + b x NIR) to produce statistics that describe the validation results.

Bias correction.The data are also examined for a bias between the methods. If the difference between means of the NIR predicted and reference values are significantly different from zero then this indicates that the calibration is biased. A bias may be removed by adjusting the constant term in the calibration equation.

Slope adjustment. If the slope (b) is significantly different from 1, the calibration is skewed.

Adjusting the slope/intercept of the calibration is generally not recommended unless the calibration is applied to new types of samples or instruments. If a reinvestigation of the calibration does not detect outliers, especially outliers with high leverage, it is preferable to expand the calibration set to include more samples.

However, if the slope is adjusted, the calibration should then be tested on a new independent test set.

Expansion of calibration set. If the error of the calibration is not meeting the expectations, the calibration set should be expanded to include more samples or a new calibration be made. In all cases when a new calibration is developed on an expanded calibration set, the validation process should be repeated on a new validation set. If necessary, expansion of the calibration set should be repeated until acceptable results are obtained on a validation set.

Changes in measuring and instrument conditions. Unless additional validation is performed, a local validation of a NIR method stating the accuracy of the method can generally not be considered valid if the test conditions are changed.

For example, calibrations developed for a certain population of samples may not be valid for samples outside this population, although the analyte concentration range is unchanged. A calibration developed on grass silages from one area may not give the same accuracy on silages from another area if the genetic, growing and processing parameters are different.

Changes in the sample presentation technique or the measuring conditions (e.g. temperature) not included in the calibration set may also influence the analytical results.

Calibrations developed on a certain instrument cannot always be transferred directly to an identical instrument operating under the same principle. It may be necessary to perform bias and slope/intercept adjustments to calibration equations. In many cases it will be necessary to standardise the two instruments against each other before calibration equations can be transferred. Standardisation procedures can be used to transfer calibrations between instruments of different types provided that samples are measured in the same way (reflectance, transmittance) and that the spectral region is common.

If the conditions are changed, a supplementary validation should be performed.

The calibrations should be checked whenever any major part of the instrument (optical system, detector) has been changed or repaired.

Statistics for performance measurement

The performances of a prediction model must be determined by a set of validation samples. This set consists of samples which are independent of the calibration set. In a plant, it will be new batches; in agriculture, it will be a new crop or a new experiment location. This set of samples must be carefully analysed following the reference methods. The care to analyse validation samples must be emphasized and the precision of these results is more important for the validation set than for the samples used at the calibration phase.

The number of validation samples must be at least 20 to compute the statistics with some confidence.

Plot the results. It is important to visualize the results in plots i.e. predicted vs. reference values or residuals vs. predicted.

The residuals are defined as:

Residue = y – x
where,
y is the reference values, determined by the standard method, and
x is the predicted values obtained when applying the multivariate NIR model.

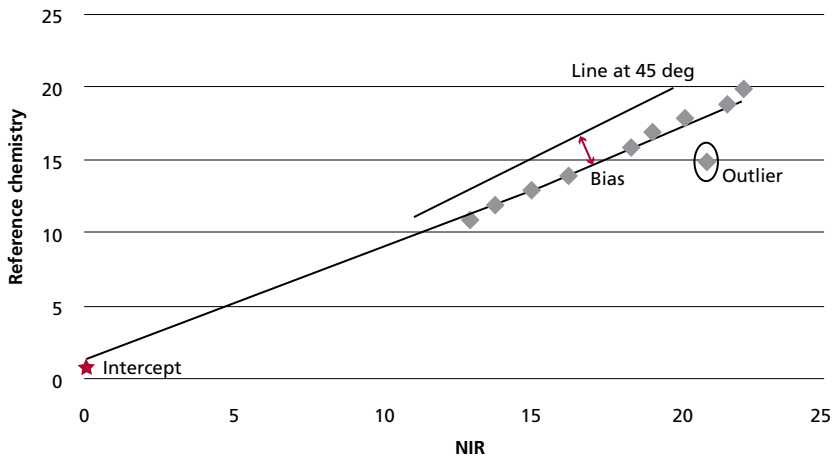
The way the differences are calculated will give a negative bias when the predictions are too high and a positive one when the predictions are too low compared to the reference values.

A plot of the data immediately gives an overview of the correlation, the bias, the slope and Intercept and the presence of obvious outliers (Figure 1).

The bias. Most of the time a bias or systematic error is what is observed with NIR models. Bias can occur due to several causes: new samples of a type not previously seen by the model, drift of the instrument, drift in wet chemistry, changes in the process, in the sample preparation. With n the number of independent samples, the bias (or offset) is the mean difference and can be defined as:

Bias = Mean (Reference Data) – Mean (NIR Predicted data)

FIGURE 1
Scatter plot for a validation set



The outlier has a strong influence on the calculation of the slope and should be removed if the results are to be used for adjustments

If the bias is significantly different from 0 then the bias value can be added to the constant value in the calibration model to correct for this systematic error.

The slope. The slope b of the simple regression y = a + bx is often reported in the NIR reports and publications.

Note that the slope must be calculated with the reference values as the dependent variable and the predicted NIR values as the independent variable, if the calculated slope is intended to be used for adjustment of NIR results. The slope can be calculated from the excel function = slope (REF values, NIR values)

If the slope is significantly different from 1 then the calibration may be adjusted according to the suppliers instructions, however the recommended procedure would be to include the validation samples into the calibration set and re-calibrate.

In order to adjust a calibration with a slope then the intercept is also required, this can be calculated from the excel function = intercept (REF values, NIR Values)

Determination coefficient (R^2). Correlation (r) indicates the degree of fit. The values provided in the validation should not be significantly different to those of the original calibration statistics. The determination coefficient (R^2) can be calculated from the excel function = RSQ (REF values, NIR values)

If the correlation is significantly different from calibration statistics then the validation samples should be added into the calibration set and re-calibrate.

Standard error of prediction (SEP). SEP provides information on the accuracy of the NIR prediction against the reference values. SEP can be calculated from the excel function =STEYX (REF values, NIR values)

If the SEP is significantly different from calibration statistics then the validation samples should be added into the calibration set and re-calibrate.

QUALIFYING (TRAINING) LABORATORY ANALYSTS

Summary

This document outlines the Standard Operating Procedure (SOP) for qualifying a Laboratory Analyst in an analytical method. There are specific stages that must be followed in a systematic order to accomplish this goal. This document serves to clarify the procedure/ criteria for qualification.

Scope

This procedure applies to all laboratory analysts and methods.

Responsibility

Laboratory Analysts investigates available methods, performs testing, generates and analyses data and demonstrates on-going competency.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data and demonstrates on-going competency.

Quality Manager specifies validation requirements and approves the method.

Procedures

Gather Information

The first step for qualifying in a new analysis is to read the SOP. A copy of this document can be obtained from the Laboratory Manager/Director.

To assure the safety of everyone, the trainee must read the Material Safety Data Sheet (MSDS) for information concerning each chemical used in the analysis. The toxicity levels and method of waste disposal should be clearly understood before beginning any analysis. If you have difficulty locating a current MSDS contact the laboratory Safety Officer.

Observe and Practice

Once familiar with the protocol and reagents, the trainee observes a qualified and competent Laboratory Analyst perform the procedure. To assure the trainee completely understands the analysis, (s) he must perform at least one practice set before beginning the actual qualifying sets. The practice set should consist of a variety of sample types and include challenging samples if available. Qualification can begin if 1) working controls meet accuracy and precision requirements in the practice set and 2) the Laboratory Manager/Director gives the trainee permission. The trainee should consult with the Laboratory Manager/Director to determine which specific reagents must be prepared for the analysis.

Run Qualifying Sets

The Laboratory Manager/Director, or their designee, selects the samples to be run by the trainee. It is preferable to use samples that have been run in duplicate and to use the average result for comparative purposes. The sets should consist of a variety of sample types. Sample target values will be provided to the trainee.

The number and types of samples analysed will be determined by the Laboratory Manager/Director. Samples may include: laboratory samples, proficiency samples, working control samples, standard reference materials and blanks. A minimum of three sets should be analysed.

Qualification Interpretation

Sufficient Number of Samples Available – Outliers will be evaluated and removed using the Dixon outlier test. The remaining data will be compared using a paired t-test. If no differences are significant ($P > 0.05$) then the Laboratory Analyst is qualified to run the analytical procedure. If there is a significant difference in the data ($P < 0.05$) then the Laboratory Manager/Director should consult with the Quality Assurance Manager.

Insufficient Number of Samples Available – The Laboratory Manager/Director should consult with the Quality Assurance Manager to develop a qualification procedure appropriate for the method.

Documentation

The Laboratory Manager/Director will submit to the Quality Manager a memo (with data attached) documenting the qualification of the laboratory analyst. This should be documented in the individual's training file.

REAGENTS AND CHEMICALS

Summary

This procedure describes the required labelling and use of bulk reagents and prepared solutions. It included the required information to be noted on reagent and preparation labels.

Scope

This procedure applies to all chemicals and reagents used in the laboratory.

Responsibility

Laboratory Analysts investigates available methods, performs testing, generates and analyses data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Procedures

General requirements

Bulk reagents, chemicals, and solutions should be properly labelled.

Expiry dates should be consistent with the guidelines and exceptions noted in the analyte SOPs.

Date expired chemicals and prepared solutions (reagents) should be properly disposed of.

No waste should be stored in the laboratory except in the designated waste container.

Bulk reagents

Bulk reagents are chemicals received from a distributor or manufacturer in the original sealed factory containers such as bottles, jars, bags, buckets, etc. Such reagents are used as received, or in preparation of solutions that are utilised in analytical procedures.

All bulk reagents must be labelled with date received and expiry information.

Bulk reagent labels must contain (at a minimum) the following information:

- Receipt date
- Initials of person receiving item
- Assigned expiration date
- Date opened
- Initials of person opening the item

All bulk reagents, regardless of container size, must have a bulk reagent label.

Some manufacturers place spaces on their labels for the information required to be on the bulk reagent label. Completing this information on the manufacturer's label does not substitute for the bulk reagent label prepared by the laboratory.

The general rule for assigning expiry dates on bulk chemicals is to use within 10 years, unless the chemical has an expiry date assigned by the manufacturer (ether, certified titrants) or as noted in the specific analyte Standard Operating Procedure (SOP).

Rotate the stock so that older chemicals are used first.

Do not stock a large quantity (more than a six-month's supply) of solvents.

If an expiry date is written as month/year, the expiry extends through the end of the

month. The expiry date of all chemicals should be reviewed no less than once a month. Expired chemicals are to be discarded: Any exceptions must be approved by the Laboratory Manager/Director. Discard chemicals via approved waste management systems only.

A Certificate of Analysis (C of A) or lot analysis is provided with some chemicals. A C of A is required for a standard reference material. A file of the certificates should be maintained in the same location as the analytical records.

Bulk reagents transferred to another container (squeeze bottles, dispensers, etc.) must contain (at a minimum) the following information:

- Reagent name
- Original Lot number from bulk container
- Initials
- Expiration Date
- Safety information if required

The following bulk chemicals can be stored in standardized pre-labelled wash bottles that can be purchased:

- Deionised Water
- Ethanol
- Acetone
- Isopropanol
- Methanol

These bottles should contain information such as the chemical name, molecular formula, target organ effects and route of entry information, CAS number and hazard warning diamonds or CLP pictogram.

Prepared solutions (reagents)

There are two requirements for labelling prepared solutions (reagents). The requirements depend on whether the solution is used within the same day it was prepared or if it will be stored for a longer period of time.

Same Day – Solutions that are prepared every day must have the following:

- Reagent name (contents)
- Concentration
- Date of preparation
- Laboratory Analyst initials
- Safety information if required

Extended Use – Reagent solutions that will not be discarded the same day must have the following information on a label:

- Reagent name (contents)
- Concentration
- Date prepared
- Analyst initials (person preparing the solution)
- Preparation number
- Expiration date
- Safety information if required

Preparation numbers are required on all solutions made in-house with the following

exception: Dilutions of a stock reference solution in order to prepare a standard curve. Preparation numbers are not required in this case, but the preparation number or information for the stock solution should be listed.

The general rule for expiration dating or solutions prepared in-house is one year. An exception to this rule includes chemicals for which the manufacturer has indicted the expiration date. Exception may also be noted in analyte procedures.

NOTE: The expiration date of the solution should not exceed the expiration of any of the components.

One format that may be used for preparation numbers is the format XXXX-YYYY, where XXXX is the laboratory notebook number and YYYY is a sequential number beginning with 0001. The preceding 0's are required so that the number is always a four-digit number.

OUTLIER TEST

Summary

If sample results are not duplicating well, an outlier test may need to be performed in order to determine if specific results can be excluded from consideration. An outlier test may be required on other sets of data, such as method qualification or method validation. The laboratory uses the Dixon outlier test (Dixon, 1995) with critical values, at 5% error, as listed in Wernimont (1995).

Scope

This standard operating procedure applies to all laboratory data.

Responsibility

Laboratory Analysts investigates available methods, performs testing, generates and analyses data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Procedures

Dixon test as applied to analytical results

For a problem sample which fails the laboratory's duplication requirement, it may be necessary to determine if a particular analytical result is an outlier. Perform the following steps to make this determination.

When there are four laboratory analytical results list the four results in order of increasing magnitude (W, X, Y, Z).

Calculate the range (R) of the values (highest – lowest) (Z – W)

Determine which result is the suspected outlier (W or Z).

Calculate the difference (T) between the suspected outlier and its nearest neighbour:

If W is suspect: $T_w = X - W$

If Z is suspect: $T_z = Z - Y$

Calculate the ratio (I):

If W is suspect: $I = T_w / R$
If Z is suspect: $I = T_z / R$

If I is greater than or equal to the critical value (N = 4) 0.829, then the suspect number is an outlier and can be excluded from consideration. If I is less than 0.829, then the suspect number is NOT an outlier and should be kept in consideration.

Dixon test as applied to other situations

The same principle above can be applied to numeric data where N is other than 4. Table 2 displays the critical values for N between 3 and 40. *NOTE:* Use a different calculation for N > 8 (see references).

If a data set is greater than 40, split the data randomly in half and test for outliers at either end of the range of results.

References

Dixon, W. J. 1953. *Processing Data for Outliers, Biometrics*. International Organization for Standardization (ISO) document ISO 5725-1981, pp. 74–89.

Wernimont, G.T. 1985. *Use of Statistics to Develop and Evaluate Analytical Methods* (W. Spendley, ed.), AOAC, 1985, Arlington, VA. Table A-9, p. 156.

LABORATORY QUALITY AUDIT CHECKLIST

Summary

The purpose of the laboratory quality audit is to systemically evaluate on-going laboratory operations in order to assure that the analytical data produced by the laboratory meets the quality criteria established for their intended use. The audit is a tool to be used to assess the effectiveness of the quality programme, to identify opportunities for improvement and to be used in training analytical staff in the requirements for sound quality policy.

It is imperative that the data generated by the laboratory be robust in terms of defensibility. Routine audits help ensure that the data generated by the laboratory are defensible, i.e. that they meet prescribed standards for precision, accuracy, traceability, completeness and comparability.

The attached checklist covers the major aspects of laboratory operations. It is in most respects a generic document. As such, it can and will evolve as needed to accommodate system and procedural changes.

This is a document designed to be used as an aid during a physical audit of the analysis of a single analytical laboratory procedure. The scope of the audit is not so limited however. Many laboratory support functions (e.g. routine equipment calibration and maintenance, safety, training, standard operating procedures, validation, etc.) are also addressed while performing the audit. An effective quality assurance program is comprised of an integrated collection of activities and the audit scrutinizes and re-evaluates each activity on a regular basis.

In preparing for the audit, the auditor should carefully read the Standard Operating Procedure for the audited analytical procedure. Having a list of details to be reviewed (reagents, equipment, standards, times, temperatures, etc.) helps focus the audit.

TABLE 2
Critical values for assessing Dixon outlier test

N	Critical Value	N	Critical Value
3	0.970	22	0.468
4	0.829	23	0.459
5	0.710	24	0.451
6	0.628	25	0.443
7	0.569	26	0.436
8	0.608	27	0.429
9	0.564	28	0.423
10	0.530	29	0.417
11	0.502	30	0.412
12	0.479	31	0.407
13	0.611	32	0.402
14	0.586	33	0.397
15	0.565	34	0.393
16	0.546	35	0.388
17	0.529	36	0.384
18	0.514	37	0.381
19	0.501	38	0.377
20	0.489	39	0.374
21	0.478	40	0.371

Generally speaking, if the answer to an audit question is no, and if there is a space for "Comments" then an explanation of the deficiency is required.

Scope

This standard operating procedure applies to all laboratory operations.

Responsibility

Laboratory Analysts investigate available methods, perform testing, generate and analyse data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Procedures

Some terminology used in the Audit Checklist (Table 3)

Auditee(s) – The responsible Laboratory Analyst(s) who performed the analysis on the audited analyte.

Auditor – the Quality Manager, or assigned deputy, who officially reviews the process and generates audit report/findings and identifies opportunities for improvement.

TABLE 3
Audit cover page

Sample Analysis Audit Checklist
Date audit was performed:
Auditor:
Sample Identification:
Analyte:
Date(s) analysed:
Auditee
Current Manager:
Responsible Manager:

List all documentation reviewed in preparing for and conducting this audit

SOP's (including version numbers): _____
Documentation relating to analytical data _____
Worksheet Reports _____
Lab Notebook #(s) _____
Balance calibration records _____
LIMS database _____
Working Control data _____
Pipette Calibration Records _____
Equipment monitoring Log(s) _____
Other: _____
Other: _____
Other: _____

Documentation

1. Is there a most-recently approved copy of the analytical standard operating procedure for the audited analyte available at the point of use for the analyst to refer to during the analysis?

No _____ Yes _____ Not Applicable _____
Comments _____

2. Is the standard operating procedure consistent with the recognised method it represents? (Non-trivial deviations from the official method must be validated before implementation.)

No _____ Yes _____ Not Applicable _____
Comments _____

3. Are all data and supporting documents immediately available and easily retrievable? All documentation relating to a particular analysis should be easily retrievable. Required data includes, but is not limited to, analytical summary sheet, assignment worksheet reports sheets, lab notebook with solution preparation information, equipment maintenance and calibration logs, computer and instrument output, control charts, etc.
No _____ Yes _____ Not Applicable _____
Comments _____

4. Does the documentation contain sufficient information and explanation such that the analysis report can be readily interpreted by knowledgeable persons other than those responsible for their generation?
No _____ Yes _____ Not Applicable _____
Comments _____

5. Does the documentation contain sufficient information such as would allow, when desirable, satisfactory repetition of the analysis under the original conditions?
No _____ Yes _____ Not Applicable _____
Comments _____

6. Are all original entries contained in the documentation neat and legible and recorded using black or blue permanent water resistant ink?
No _____ Yes _____ Not Applicable _____
Comments _____

7. If the procedure used to analyse the sample is not a recognised procedure was the procedure validated?
No _____ Yes _____ Not Applicable _____
Comments _____

8. If the procedure used to analyse the sample is a validated procedure, is the validation documentation archived and readily available?
No _____ Yes _____ Not Applicable _____
Comments _____

9. If there are any corrections or changes to any entries in the documentation were they properly made? Proper corrections are made as follows:

- No erasures, correction fluid or correction tape may be used while making corrections to any documents.
- Draw a single line through the incorrect entry; do not obliterate the original entry – it should be legible through the strikeout.
- Write the correction in as close a proximity to the original as possible.
- The analyst's initials should appear near the correction.

- The date of the correction should appear near the correction (unless a date is being corrected, in which case the correction dates itself!).
- If the reason for the correction is not obvious, then there should be a brief explanation of why the change was necessary. The explanation should be written near the correction if possible, otherwise it can be referenced elsewhere on the page or attached sheet using an "*".

No _____ Yes _____ Not Applicable _____
Comments _____

10. Does the Analytical Summary Sheet provide for recording the residence times, temperatures, digestion times, bath temperatures, etc. for all critical steps defined in the standard operating procedure?
No _____ Yes _____ Not Applicable _____
Comments _____

11. Are all weights below 1 gram in the form 0.xxxx (including preceding zero)?
No _____ Yes _____ Not Applicable _____
Comments _____

12. If inserts are used (i.e. paper such as chromatograms, printouts, etc.) are they permanently attached (stapled, taped) or placed in a separate envelop (fully cross-referenced to the original set and easily retrievable)?
No _____ Yes _____ Not Applicable _____
Comments _____

13. During the analysis was any instrument output generated? Are all entries on instrument output properly labelled (e.g. concentrations of calibration standards, ID of working standards, individual sample numbers for investigative samples, sample dilutions [if different than described in the standard operating procedure], etc).
No _____ Yes _____ Not Applicable _____
Comments _____

14. Is the computer database information consistent with paper documentation?
No _____ Yes _____ Not Applicable _____
Comments _____

15. If any results were generated using external software (i.e. a spread sheet program), was the external program validated before use, and is the validation documentation archived and readily available?
No _____ Yes _____ Not Applicable _____
Comments _____

16. Are any comments, if given, reasonable, complete, and supportive of data observations within the set documentation?
No _____ Yes _____ Not Applicable _____
Comments _____

17. Was the set properly reviewed by the reviewer?
No _____ Yes _____ Not Applicable _____
Comments _____

18. Are all required blanks either filled in or correctly marked through?
No _____ Yes _____ Not Applicable _____
Comments _____

19. Is each page of the documentation consecutively numbered in the format "m/n" or "m of n", where m is equal to the page number and n is equal to the total number of pages? (Continuous, i.e. un-separated, computer printouts and self-paginated spread sheet and instrument printouts are considered one page).
No _____ Yes _____ Not Applicable _____
Comments _____

20. Is the set identification number included on all required pages of the documentation?
No _____ Yes _____ Not Applicable _____
Comments _____

21. Is each required page of the documentation signed and dated by the responsible analyst?
No _____ Yes _____ Not Applicable _____
Comments _____

Reagents and solution preparation

22. Were all volumetric and test solutions used before their respective expiration dates?
No _____ Yes _____ Not Applicable _____
Comments _____

23. Are unique identification numbers for all bulk reagents, volumetric solutions, and test solutions used during the course of the analysis included on the Analytical Summary Sheet?
No _____ Yes _____ Not Applicable _____
Comments _____

24. Are all unique identification number for bulk reagents, volumetric solutions, and test solutions in the required format and recorded in the laboratory notebook?
No _____ Yes _____ Not Applicable _____
Comments _____

25. Were all bulk reagents used in the preparation of solutions for the audited analysis used before their respective expiration dates?
No _____ Yes _____ Not Applicable _____
Comments _____

26. Are the preparation dates in the laboratory notebook for each volumetric and test solution consistent with the dates of use indicated on the Analytical Summary Sheet?
No _____ Yes _____ Not Applicable _____
Comments _____

27. Are the unique identification numbers on the current volumetric and test solutions consistent with the preparations described in the laboratory notebook?
No _____ Yes _____ Not Applicable _____
Comments _____

28. Are all samples, extracts, reagents, reference materials and standards stored so as to preserve their identity, concentration, purity and stability?
No _____ Yes _____ Not Applicable _____
Comments _____

29. Are all calibration standard or reference standard materials used in this analysis traceable to a (primary) Certified Reference Material such as NIST or USP?
No _____ Yes _____ Not Applicable _____
Comments _____

Laboratory notebooks

30. Is each separate reagent or solution preparation on a given page of the laboratory notebook initialled and dated by the analyst, and does the description include the following?

- A unique identification number for each prepared solution.
- The name/concentration of the volumetric or test solution being prepared (e.g. 0.15 N HCl in methanol).
- The name (and molecular formula if more than one form is possible) of the bulk reagent(s) being used in the preparation of the volumetric or test solutions.
- Weights [(gross, tare, net) or (weight by difference)] or volumes, expressed in appropriate units, of the bulk reagent used in the preparation of the volumetric or test solution.
- The sequence of dilutions (if necessary) used to prepare the final concentration of the volumetric or test solution, including volumes taken and final volumes.
- The unique identification of the balance, pipette, or dispenser used.
- Expiration date of the prepared solution.
- Identification of the water source.

No _____ Yes _____ Not Applicable _____
Comments _____

Instrumentation and equipment

31. Does the Analytical Summary Sheet provide an entry to record the unique ID number of each critical piece of equipment that was used in the analysis?
(i.e. balance, oven, pipette, dispenser, thermometer, titrator, refrigerator, etc.)?
No _____ Yes _____ Not Applicable _____
Comments _____

32. Of the listed equipment requiring calibration or maintenance, was each properly performed?
No _____ Yes _____ Not Applicable _____
Comments _____

33. Is the daily or weekly balance calibration for the balance used in the audited analysis documented for the day of the analysis? Is the balance in-control? Are all associated calibrations in-control?
No _____ Yes _____ Not Applicable _____
Comments _____

34. Are any daily temperature recordings out-of-control for any listed equipment requiring daily monitoring?
No _____ Yes _____ Not Applicable _____
Comments _____

Control charts

35. Are the working control data for the set located on the Analytical Summary Sheet, and is this data in-control for the audited analyte? If there is a trend in the previous year's working control data, has it been addressed in the control chart notepad?
No _____ Yes _____ Not Applicable _____
Comments _____

36. Is the control chart database up-to-date for the analyte being audited?
No _____ Yes _____ Not Applicable _____
Comments _____

37. Have the control chart statistics been correctly calculated? Is it documented properly?
No _____ Yes _____ Not Applicable _____
Comments _____

Health & safety

38. Are all personnel encountered during this audit wearing their personal protective equipment?
No _____ Yes _____ Not Applicable _____
Comments _____

39. Are special storage or handling requirements indicated on the analytical documentation?
No _____ Yes _____ Not Applicable _____
Comments _____

40. Are current samples requiring the same analysis being properly stored/handled?
No _____ Yes _____ Not Applicable _____
Comments _____

41. Is the analyst who is currently assigned to perform the audited analysis equipped with appropriate safety equipment (i.e. lab coat, gloves, reinforced toe shoes, face shield, dust mask, vapour mask, rubber apron, etc.) as necessary?
No _____ Yes _____ Not Applicable _____
Comments _____

42. Does the current analyst know the location of the nearest eyewash? fire extinguisher? fire blanket? spill clean-up kit? fire alarm? emergency exit? emergency phone? emergency shower? Are they all working and in-date?
No _____ Yes _____ Not Applicable _____
Comments _____

Miscellaneous

43. Are any deviations from the protocol apparent from either the audited documentation or from interviewing the analyst(s)? Explain
No _____ Yes _____ Not Applicable _____
Comments _____

44. If there were any deviations from the protocol, were they approved by the supervisor and explicitly documented?
No _____ Yes _____ Not Applicable _____
Comments _____

45. What was the Z-value on the last check sample and were the results in control?
No _____ Yes _____ Not Applicable _____
Comments _____

46. Is there a trend in the previous year's check sample control charts?
No _____ Yes _____ Not Applicable _____
Comments _____

RECEIVING LABORATORY SAMPLES

Summary

This section identifies the procedures used to handle samples submitted to the laboratory, from collection and shipment by clients, to receipt.

The sample chain-of-custody within the laboratory begins when samples are physically received. Specific handling requirements are dictated by the nature of each sample; see "Handling of Feed Samples". The Laboratory expects fully trained field inspection staff to conduct effective sampling in order to provide representative and consistent laboratory samples for evaluation. The extent to which the sample represents the parent population is critical if the data returned to the client is to serve any useful purpose.

Scope

This procedure applies to all laboratory feed and feed ingredient samples submitted to the laboratory for testing.

Responsibility

Laboratory Analysts investigate available methods, perform testing, generate and analyse data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Sample Technicians become proficient in receipt, handling, and documentation of samples.

Procedures

Examine Condition of Sample

Each laboratory sample must arrive in good physical condition and in a suitable clearly labelled container and be accompanied by an appropriate sample submission form. Note sample state on arrival i.e. frozen, chilled, room temperature etc.

Accept/Reject Sample

Sample rejection may occur due to physical deterioration, potential contamination due to leakage, broken container, mingling of samples shipped together, insufficient sample for the required test(s) and improperly shipped or identified samples.

Login or Document Sample

Unacceptable samples are referred to the appropriate supervisor, who notifies the Client to discuss the corrective action required. The sample may still be analysed, if requested by the Client, but all reports generated with such samples will include a statement indicating their receipt in an unacceptable condition and will describe the nature of the problem. Samples which are not to be processed are logged into the system and reported to Clients as "unfit". A comment relating to the reason for declaring the sample "unfit" can be added to the sample identification document and to the report of analysis. See Table 3 for sample login form.

Identify Sample

A unique sample identifying number is assigned – The Laboratory Sample Number is recorded on the laboratory sample (Table 4), documents and test samples and is used in the laboratory to track the sample throughout the testing process, i.e. samples, storage, containers, retained sample, reports of analysis, documents, worksheets and work books.

Appendix

Date: _____

Received by: _____

NOTE: Circle appropriate Status Code beside each Sample Number

TABLE 4
Sample arrival status

Sample Number	Status Code
1	A B C D E F G H J K
2	A B C D E F G H J K
3	A B C D E F G H J K
4	A B C D E F G H J K
5	A B C D E F G H J K
6	A B C D E F G H J K
7	A B C D E F G H J K
8	A B C D E F G H J K
9	A B C D E F G H J K
10	A B C D E F G H J K
11	A B C D E F G H J K
12	A B C D E F G H J K

A Sample in acceptable condition
B Sample container broken, torn, or leaking
C Custody seal broken
D Custody seal missing
E Sample contaminated by leaking sample
F Sample wet, possibly contaminated
G Insufficient sample quantity
H Sample received without paperwork
J Paperwork received without sample
K Other issues

HANDLING AND PREPARATION OF FEED SAMPLES

Summary

This protocol describes the proper handling/grinding/preparation of feed samples by the analysts in the laboratory. Specific handling requirements are dictated by the nature of each sample. The sample chain-of-custody within the laboratory begins when samples are physically received.

Scope

This procedure applies to all laboratory feed and feed ingredient samples.

Responsibility

Laboratory Analyst investigates available methods, performs testing, generates and analyses data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Sampling

Sampling can be one of two types; Representative Sampling or Selective Sampling.

Representative Sampling

Representative Sampling obtains a small fraction from a larger volume in such a way that a determination of a required characteristic will represent the mean value of the characteristic of the entire sample.

Selective Sampling

If a noticeable difference is observed in a portion of the sample to be analysed this portion shall be separated from the entire sample and treated as a separate lot. If this is not possible the entire sample shall be processed and the proportion of sample which had a noticeable difference recorded.

In either case details shall be recorded in the final report to the client.

Statistical Consideration

Acceptance sampling is the usual method of sampling for animal nutrition laboratories. For sampling by attributes, there is a theoretical sampling plan based on binomial distribution. This plan has been simplified to a square root relationship between lot size and the number of increments.

With bulk product sample variances are expected to be uniform if, for lots of up to 2–5 tonnes at least seven increments are taken and, for between 2–5 and 8 tonnes the number of increments taken is at least equal to $\sqrt{20}$ m (where m = mass in tonnes of the sample). If the lot exceeds 80 tonnes, the square root relationship is still applicable but will be less accurate.

Equipment

Grinders or Mills. These should be capable of grinding feedstuffs without causing any noticeable changes in moisture in the sample and should not generate excessive heat (which may have a detrimental effect on the sample). If the feedstuff to be analysed is likely to lose or gain moisture a correction factor should be applied to the results. This is determined by comparing the moisture content of the prepared (ground or milled) sample against a portion of the original sample before processing. Once ground or milled the sample should be suitable to pass through an appropriate sieve. Grinders or mills should be thoroughly cleaned after use to avoid cross contamination.

Riffler/Homogeniser (or mechanical stirrer), to homogenise moist feedstuffs. A mincer with a 4 mm plate may also be useful for frozen feedstuffs.

Sieves, of aperture size 1.0 mm, 2.8 mm and 4.0 mm made from woven metal wire cloth or similar.

Analytical Balance, used to determine the sample weight (see Quality Procedure Section 8 'Use of Balances').

Mechanical Shaker, used to shake viscous liquid molasses sample.

Dividing or Quartering Apparatus, equipment such as a conical divider or multiple slot divider with a sorting system will ensure uniform dividing of laboratory samples.

Tools, hammer, used to hit screwdriver/chisel.

Screwdriver/chisel, used to break molasses block into smaller pieces.

Spatula, used for scooping samples, *mortar and pestle* for crushing samples.

Transfer pipette, used in the delivery of wet feed and liquid feed.

Sample Containers, these should be suitable for preserving the sample integrity avoiding any changes or effects from moisture, temperature or light. The sample container should be of suitable size to allow storage of sufficient sample to complete all determinations required (not less than 100 g) and there should be some air space left once filled (to allow effective mixing prior to sampling for all tests required). The container should have a securely fitting lid and should be uniquely identified with a sample identifier on the container and not the lid.

If a sample is to be examined microbiologically it should be handled under sterile conditions to preserve the microbiological load.

Procedure

To avoid exposure to the atmosphere grinding or milling should be as rapid as possible. It may be necessary to break or crush the sample prior to using the grinder or mill.

Fine samples

If a sample can pass through a 1.0 mm sieve it should be mixed thoroughly and divided successively using dividing or quartering apparatus (see 7.)

Coarse samples

If the sample does not pass through a 1.0 mm sieve but passes through the 2.8 mm sieve it should be ground until it does pass through the 1.0 mm sieve and divided as for fine samples. Likewise if a sample passes through the 4.0 mm sieve but not the 2.8 mm sieve.

Difficult to grind samples

If a sample is difficult to grind using the grinder or mill the moisture content of a subsample from the initial sample should be determined and the remaining sample crushed with a mortar and pestle until it passes through the 1.0 mm sieve. The moisture content of the crushed sample should then be determined to allow a correction factor to be assigned.

Grass or cereal silage

The entire sample should be milled and mixed. Some samples may require chopping finely beforehand. If the sample is unsuitable for milling or chopping the sample may be dried in an oven overnight (60–70 °C) and milled. The moisture content should be determined and a correction factor applied.

Liquid samples

The sample should be mixed using the homogeniser or stirrer to ensure complete dispersal of separated material and the sample transferred using a wide bore pipette.

Troubleshooting

A false high/low result for a specific analyte and/or poor duplication may be attributed to one or more of the following:

1. Incomplete or inconsistent mixing of dry, liquid and wet samples prior to weighing.
2. Not allowing refrigerated or frozen samples to reach room temperature prior to weighing.
3. Not gathering portions of a sample from different locations around the molasses/lick block prior to weighing or not analysing a large enough portion of the sample (sample weight may need to be increased for consistent duplication).
4. Samples must be prepared in such a way that the amounts weighed out as provided for in the methods of analysis are homogeneous and representative of the final samples.

Sample Handling

Dry Feed Sample Preparation

1. Riffle the sample. Generally, riffle the original sample until a satisfactory sized sub-sample is obtained. Place the riffled, unground sample in a labelled Ziplock (or similar) bag and the other portion(s) of unground sample back in the sample bag to be retained. Label each container (bag, bottle, etc.) with a matching identification number (e.g. a barcode).
2. Grind one of the portions (bag portion) through the appropriate mill.
3. Grinding or milling of some samples may lead to a loss or gain of moisture and volatile matter, allowances should be made for this. Grinding should be as rapid as possible to avoid exposure to the atmosphere.
4. Place ground sample on brown paper.
5. Thoroughly mix the sample before taking the laboratory portion
6. Fill a laboratory sample bottle with sufficient ground sample by passing through the mixed sample.
7. Return to the sample storage room immediately after analysis.

Procedure for Obtaining Laboratory Sample

1. Allow sample to warm to room temperature if stored in a freezer or refrigerator.
2. Mix the sample by rolling the bottle and tilting the bottle from the left to right (twirl) for several seconds. Do not shake.
   Some samples may need additional mixing (e.g. 41% cottonseed samples).
3. Weigh sample by means appropriate for the desired method.
4. Return the sample to the appropriate storage area.

Liquid Molasses Samples

1. Samples should be stored refrigerated.
2. Allow sample to warm to room temperature.
3. Shake sample vigorously for 1 minute to provide thorough mixing. For those samples that are too viscous to be shaken by hand, a mechanical shaker needs to be used. Shake the sample for no less than 15 minutes or as described in the method protocol to provide thorough mixing.
4. Weigh sample by means appropriate for the desired method. (Clean all spillage from the outside of the bottle).
5. Return the sample to the appropriate refrigerator.

Molasses/Lick Block Samples

1. Samples should be stored refrigerated.
2. Allow sample to warm to room temperature.
3. For soft blocks using a spatula cut off small portions of the sample from different locations around the block until the desired weight is achieved. Mixing is not practical.
4. For hard blocks chisel off small portions of the sample from different locations around the block until the desired weight is achieved. (Use a hammer and screwdriver/chisel, ensure appropriate personal protective equipment is used).
5. Return the sample to the refrigerator.

Samples for mycotoxins

1. Grind entire sample (Romer Mill or equivalent mill). *NOTE:* The Romer Mill will separate the sample into two unequal portions. One portion will be approximately $^1/_3$ and the other portion will be approximately $^2/_3$ of the whole sample.
2. Grind the $^1/_3$ portion through a 1.5 mm sieve using appropriate grinder (e.g. Retsch SR 300, Germany or equivalent grinder).
3. Pour ground portion onto brown paper and four corner mix.
4. Clean the grinders with compressed air or grind small amount (200 g) of uncontaminated grain.

References

ISO 6497. 2002. *Animal feeding stuffs – Sampling*. Geneva, Switzerland.

EN ISO 6498. 2009. *Animal feeding stuffs – Guidelines for sample preparation*. Geneva, Switzerland.

USE OF BALANCES

Summary

This document provides the procedures to calibrate, clean, weigh materials, and perform routine maintenance on top-loading and analytical laboratory balances.

Scope

This standard operating procedure applies to all Laboratory Analysts using top loading and analytical balances.

Responsibility

Laboratory Analysts investigates available methods, performs testing, generates and analyses data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Equipment

Weight set, stainless steel, NIST/NVLAP or similar certified yearly
0.1 g, 1 g, 10 g, 50 g, and 100 g range

Procedures

Cleaning

Cleaning is essential in everyday usage of the balances to maintain quality assurance in the laboratory since weighing is the most basic and yet one of the most important steps in an analysis. It is inevitable for the balances and weights to become dirty with everyday use. Therefore, it is important to follow the proper methods carefully to obtain the most accurate results. The following methods for cleaning the balances and weights were developed to ensure quality results.

Unplug the balance before cleaning and do not use any harsh or abrasive cleaning agents. Allow nothing to enter the balance's internal mechanism. Also handle the balance pan with care, do not touch the balance pan feet on which it rests or the place where the pan contacts the balance. When cleaning the weighing pan with a liquid solution, remove the pan and clean it outside of the balance to prevent any liquids from running into the internal electronics and causing damage. If some material enters the balance housing, notify a supervisor immediately so proper care can be taken to limit the damage to the balance.

The balance should be cleaned as necessary. For simple cleaning, such as removing sample from the balance pan, a soft camel hair brush should be used. Make certain no small particles remain on the balance pan since even the smallest amount can affect a reading or corrode the pan.

If the balance pan cannot be cleaned simply by using a brush, then water may be used to wipe clean the weighing pan. A lint-free wipe should be utilized to wipe away any dirt and water on the pan. The water should be put onto the wipe, not on the balance.

If necessary, a 1% soap solution may be used to help clean the weighing pan, but this must be used with care. The soap solution must be thoroughly washed off. Soap tends to

leave a residue on the balance, therefore affecting the results of a weighing. A lint-free wipe should be used to wipe off the balance pan.

For the analytical balances, the windows may be cleaned with a glass cleaner and a lint-free wipe. The glass cleaner should be sprayed onto the wipe and then applied to the windows. This will prevent any build up from the spray.

The weights should never be handled without the use of a lint-free wipe or tweezers. For the larger weights, powder-free gloves may be used when necessary. Fingerprints will affect the results of a weighing and will decrease the accuracy and the precision.

To clean the weights use ethyl ether and a lint-free wipe. The ether should only be used in a solvent hood. Wipe the weight until it is visually clean and no ether remains. The weights should be cleaned as needed.

Calibration

Calibration is an important factor in assuring quality results. Without a proper calibration, the balance is unable to produce results which are viable and consistent. Therefore, it is essential to routinely check the calibration of a balance. There are three methods which will be incorporated in order to establish this routine.

First of all, the balances are to be checked and calibrated by a certified service technician annually. The annual maintenance records should be maintained in a balance logbook along with the weekly calibration records. The maintenance should be recorded with the date, company, technician and reason for maintenance. Calibration weights should be checked at this time also. Any correction factors assigned should be recorded and used until the next calibration

Secondly, every week the Laboratory Analyst, or their designee, will check the calibration of the balance in his/her respective lab with nationally traceable rated weights. The weekly calibration records should include: descriptive title, name, manufacturer, model number, serial number, room number, required calibration frequency, allowed "working" limits, initial and date of calibration and responsible Laboratory Analyst review. The weights must be within the accuracy range that will be determined for each balance.

Finally, on a daily basis and prior to the calibration check, an internal calibration will be performed. Individual balance manuals and calibration protocols should be followed for specific calibration techniques. These should be recorded on the appropriate document.

Some balances have an internal weight that is utilized to calibrate the balance. Other balances need an external weight to be calibrated. Refer to balance manuals for the specific procedures for each balance.

Calibrating Top Loading Balance

The following steps are to be followed weekly to check the calibration of the top load balances. A calibration that approximately spans the weighing range should be done using the nationally traceable weight set, with a single calibration reading for each weight. Before calibrating, make certain the balance is clean. Do not touch the weights directly with your hands. Use a dust-free glove, tweezers or a lint-free wipe to handle the weight (see cleaning protocol).

Check the levelling bubble to make sure the balance is level. If it is not, level the balance

by adjusting the balance feet. The level bulb is usually located at the rear of the balance for top load balances. Turn the levelling feet clockwise to raise the balance and counter-clockwise to lower the balance. The balance cannot be moved and then used without a readjustment or check of the level.

With the balance pan empty, press the tare button. This should result in a reading of 0.00.

Place a clean weight on the weighing pan by using a lint-free wipe or a pair of tweezers. Record the value on a calibration form. If the range is not within the limits, discontinue use of the balance and investigate the problem (see Balance Ranges and Limits below). If your balance has a calibration function, an internal calibration can also be done using the specified weights (Class S) listed in the balance manual.

Calibrating Analytical Balance

The following are the steps to be followed weekly to properly check the calibration of the analytical balances. A calibration that approximately spans the weighing range should be done using the traceable weight set. Before calibrating, make certain that the balance is clean. Do not touch the weights directly with your hands. Use a dust-free glove, tweezers or a lint-free wipe to handle the weight (see cleaning protocol). Make certain the doors are closed before calibration and during weighing to prevent air currents from affecting the readings.

Check the levelling bubble to make sure the balance is level. If it is not, level the balance by adjusting the balance feet. The level bulb is usually located at the rear of the balance. Turn the levelling feet clockwise to raise the balance and counter-clockwise to lower the balance. The balance cannot be moved and then used without a readjustment or check of the level.

Make sure the balance pan is empty. Press the tare button; the output should be either 0.0000 or 0.00000.

Analytical balances are capable of using their internal weights for calibration. If your balance has a calibration button, press the button to calibrate. The reading will progress from C to CC. Once it is finished, the reading will return to 0.0000. If your balance has a knob for calibration, turn the knob slowly until a C appears. Continue turning the knob until the knob is to the calibration position. The reading will display CC when the calibration is finished. The Laboratory Analyst must return the knob to its original (or middle) position before weighing; the display will again read 0.0000. See balance manual for instructions pertaining to other models.

Place a clean weight on the weighing pan by using a lint-free wipe or a pair of tweezers. Record the value on a calibration form. If the range is not within the limits, discontinue use of the balance and investigate the problem (see Balance Ranges and Limits below).

Balance Ranges and Limits

The weighing range indicates the minimum and maximum amounts the balances can weigh. See individual balance manuals for specifications.

The accuracy limit is used for the weekly calibration checks. The limit indicates the amount that the weight can deviate from the certified amount. Typical limits are listed below:

Top Loading Balance Weight	< 100 g ± 0.10 g ; > 100 g ± 0.50 g
Analytical Balance Weight	± 0.0005 g

Corrective Actions for Balance Problems

If the weekly balance check is outside the limits, the balance should be re-examined. Clean, check the level, and recalibrate. Check for draughts or other conditions that may affect the balance. Check the calibration again with the weight. If it passes, record comments on the log sheet. If the check fails in all weight ranges, the balance should be placed out of service by the Laboratory Analyst and labelled as such to prevent use. If the check passes at the lower weight ranges, the balance should only be used for weighing in the lower ranges and a warning sign should be applied to the balance.

For any balance problems, notify the analyst and supervisor so that the balance can be repaired or replaced.

Interferences

There are several anomalies that may occur during the standard operation of the balances. A common occurrence is balance drift which can arise from several different factors. To check the balance for drift, tare the balance and see if the readout remains steady.

The most likely cause of balance drift is an improperly levelled balance. It is very important before each use of the balance that the level bubble is checked and the balance feet are adjusted if not properly aligned.

Balance drift also will occur if the doors on the analytical balance are left open during weighing. The doors must be tightly shut; air currents are capable of affecting the final weight. The effect of air currents on a reading varies depending on the conditions of the room in which the balance is used.

Vibrations in a room also result in balance drift. This problem is dependent on the room and building in which the balance is located.

Occasionally it is necessary to place a balance in a location that has a severe amount of vibrations. For example, when weighing toxic substances, it is necessary to weigh the substance in a fume hood. The problem of balance drift can often be resolved by placing the balance on a marble stand. Even if the balance is not in a fume hood, it is wise to place an analytical balance on a marble stand to protect the balance from slight movement caused from building vibrations or accidentally bumping the counter where the balance is placed.

When a balance is moved from one room to another, the balance should be allowed to equilibrate in the new environment for about two hours in order to stabilize to ambient temperature conditions. Do not move a calibrated balance. If the balance is not in thermal equilibrium with the environment then balance drift will occur. When moving a balance from a cooler room to a warmer room, allow the balance to equilibrate for 2 hours unplugged to avoid condensation of moisture on or in the balance. Variations in humidity and temperature cause drift.

One way to resolve the problem of high humidity is by placing an AIR-DRYER (silica gel desiccators created for balances) on the inside of the balance to remove any moisture. An ideal location for the balance is a room with air conditioning 24 hours a day. Optimum conditions include avoiding exposure to extreme radiation of heat and aggressive chemical atmospheres.

Weighing tips

Do not drop the object to be weighed onto the balance pan, this will damage the internal structure and the weights may no longer be accurate.

Do not leave a sample sitting on the weighing pan for extended periods of time. Also, make certain the sample and the balance are at the same temperature. If the sample is cold, condensation may form while weighing and the wrong amount will be measured. Be certain to re-tare the balance after use.

When there is a power outage, it is necessary to allow the balance to warm up for one hour.

When placing an object on the balance, allow the balance to equilibrate (display 'g') before continuing. Once the 'g' appears a weight can be taken or the scale can be tared.

USE OF PIPETTES

Summary

This document provides the laboratory procedures to be used with pipettes and dispensers. Optimum calibration conditions, calibration mediums, calculations for pipette specifications and problem solving are included in this protocol. There are three types of pipettes described in this protocol. One is the hand-held pipettor, like the Eppendorf and the Pipetmann. A Brinkmann or Repipet is attached to a bottle, and is known as a dispenser. The third type, such as a Brinkmann Dosimat, has an attached dispensing unit. "Pipette" is used to describe all of these unless otherwise specified. Dispensers may not need to be calibrated but should be tested to ensure fit for purpose. Dosimats and hand held pipettors should be serviced and calibrated by a service technician on a yearly basis and documented in the appropriate equipment register.

Scope

This standard operating procedure applies to all Laboratory Analysts using laboratory pipettes and dispensers.

Responsibility

Laboratory Analyst investigates available methods, performs testing, generates and analyses data.

Laboratory Manager/Director evaluates and authorises methods, reviews and approves data.

Quality Manager specifies validation requirements and approves method.

Equipment

Analytical balance
Pipettes or dispenser
Thermometer

Procedures

Calibration

Before calibration, the pipette should be rinsed with water, or other weighing medium, to remove all of the previous solution. The Dosimat unit should also be rinsed with the

weighing medium before calibration. Optimum conditions for the calibration measurements involve the type of weighing vessel, the environment, the testing medium, and the Laboratory Analyst's technique. The calibration weighing vessel should be cylindrical in order to keep the surface of the liquid sample constant. To minimize evaporation, the container should be covered. To minimize fingerprints, use tweezers, gloves or lint-free wipes when handling the weighing vessel. The room that the measurements take place in must be draft free and have no direct sunlight on the balance. The room must be normal ambient temperature (19–23 °C) or allow the pipette to equilibrate to room temperature before calibrating. The Laboratory Analyst's technique is important, follow the pipette's instruction manual and keep the timing between weights consistent.

For most pipettes, the testing medium is deionised water. It is necessary that a portion of the medium be placed into the sampling container to equilibrate for one hour prior to calibration. It is essential that the water temperature be measured to the nearest 0.1 °C for future calculations (see Table 5).

There may be occasions when the pipette should not be calibrated using water. These types of pipettes should be calibrated using a compatible solvent. If the compatible solvent is highly volatile, a medium is dispensed into the flask, the top is replaced, and the flask is reweighed to get the final weight. When a solvent is used, the density (g/ml) of the solution must be taken into account, this will replace the Z-factor used in a water calibration. To find the density, the room temperature must also be taken.

At a minimum, pipettes that are used on a regular basis must be calibrated monthly with three sets of 5 weighings. The frequency may be reduced if a low risk is identified. For pipettes that are used less frequently, calibrate the pipette with each use. This determines the accuracy and the precision. It is also necessary to calibrate with three sets of 5 weighings after major service is completed or when a new instrument has arrived. The Dosimat exchange unit should be calibrated once a year by the instrument manufacturer.

These weighings are to be done at three different pipette volumes. The weighing must span the range of use for the pipette in the laboratory. For example, a 10 ml pipette would be tested at 2 ml, 5 ml and 10 ml volumes. The pipette may also be calibrated at a reading commonly used in the lab. For example, if a 9 ml measurement is needed every day, the pipette can be calibrated at 2 ml, 5 ml and 9 ml. The analyst needs to determine the three measurements to be taken based on the needs of their laboratory. If a pipette is set to only dispense a specific volume, then the calibration only needs to be done at that volume. If a pipette is used only to fill to volume or to dispense a non-critical volume, it need not be calibrated. These special pipettes must be labelled to inform analysts of the pipette's status.

Specifications

Specifications are determined from the measurements taken during the calibration. If the pipette does not meet the required specifications it should be examined in-house or be sent to the manufacturer for recalibration or be replaced. When the pipette does not meet specifications it must also be labelled with an out of service sticker. The specification calculations are as follows.

The sample weight is converted to sample volume by multiplication of the Z-factor (ml/g). However for pipettes that must be calibrated with a compatible solvent, the density

TABLE 5
Z Factors for converting sample weight to sample volume

Water Temperature (°C)	Z-Factor (ml/g)
15	1.002
15.5	1.002
16	1.0021
16.5	1.0022
17	1.0023
17.5	1.0024
18	1.0025
18.5	1.0026
19	1.0027
19.5	1.0028
20	1.0029
20.5	1.003
21	1.0031
21.5	1.0032
22	1.0033
22.5	1.0034
23	1.0035
23.5	1.0036
24	1.0037
24.5	1.0038
25	1.0039
25.5	1.004
26	1.0041
26.5	1.0042
27	1.0043
27.5	1.0044
28	1.0045
28.5	1.0046
29	1.0047

is used, and the weight must be divided by the density (g/ml). Make certain to use the correct solvent density based on the lab's temperature. A list of Z-factors is located in Table 4. The density can be obtained through the CRC, Handbook of Chemistry and Physics.

$$\% \text{ Error (or Accuracy)} = \frac{(\text{Mean Volume} - \text{Theoretical Volume}) \times 100}{\text{Theoretical Volume}}$$

Precision = Standard Deviation = Repeatability

% CV (Coefficient of Variation) = (Standard Deviation)/(Mean Volume) x 100

The specifications or calibrations limits, for a given pipette are determined by its use in the laboratory. There are three types of use in the laboratory; one is critical, one non-critical and one not needing to be calibrated. Some dispensers are used to fill to volume, and for such uses, do not require calibration. Only the manager has the authority to change limits. Suggested limits for critical and non-critical use are listed below.

Critical:	Accuracy: ≤ 2.0%
	Precision: ≤ 0.75%
Non-Critical:	Accuracy: ≤ 6%
	Precision: ≤ 2.5%

Troubleshooting

Inaccurate volumes can occur if the proper pipetting procedure is not followed such as, the tips do not properly fit the pipette being used, or they are not placed tightly onto the pipette. These problems can be determined by observing the placement of the ejector arm on the pipettor. If it interferes with pipette operation, something is wrong. With all pipettes and dispensers, a loosened or cracked shaft will affect the volumes delivered. Any problems with the internal mechanisms of the pipette should be referred to an experienced repair technician. Sample volume can also become inaccurate if the sample is splashed into the pipette.

Care of Pipettes

Must be stored upright, do not leave lying on bench. If using corrosive or large volumes, use filters.

OPERATION OF PH METER

Summary

This standard operational procedure describes the method used to calibrate and use laboratory pH meters.

Scope

This standard operational procedure applies to general use pH meters.

Reagents

- Water, distilled
- pH buffer 4.00 ± 0.01
- pH buffer 7.00 ± 0.01
- Potassium chloride 3 M – weigh 224 g KCl into a 1L volumetric flask and fill to volume with deionised water.

Equipment

- pH meter
- Combination electrode
- Beakers
- Stir plate
- Stir bars
- Water bottle

Procedures

Calibration (Daily)

- Pour fresh pH 4 and pH 7 buffer into small beakers (retain calibration certificates for buffers)
- On the pH meter turn the setting to pH
- Rinse the electrode with deionised water
- Place the electrode in the pH 7 buffer. Stir the buffer on a stir plate or swirl by hand.
- If necessary, adjust the pH to 7.00 using the calibration knob.
- Remove the electrode from the pH 7 buffer.
- Rinse the electrode with deionised water.
- Place the electrode in the pH 4 buffer. Stir the buffer on a stir plate or swirl by hand.
- If necessary, adjust the pH to 4.00 using the temperature knob.
- Remove the electrode and rinse with deionised water; keep the electrode in a beaker of deionised water during use and store in 3 M KCl after use.
- After use return the setting to standby.
- Discard used buffer.

Use

- Place electrode in solution to be measured. The solution can be stirred if desired.
- Once pH reading stabilizes record the pH.
- Remove the electrode and rinse with deionised water.
- Repeat for each sample, electrode can be kept in deionised water between samples.
- When finished store electrode in 3 M KCl and return the setting to standby.

OPERATION OF SPECTROPHOTOMETER

Summary

Spectrometry is a technique used to measure how much radiant energy a substance absorbs at varying wavelengths of light. The wavelength at which peak absorption occurs is useful when trying to identify an unknown. By measuring the absorption spectrum of a substance it is possible to identify or place a substance into a class of compounds. The Beer-Lambert Law describes the relationship between absorbance and solute concentration and the length of the light path. Absorbance is directly proportional to the concentration and length of the light path. Therefore, a plot of absorbance versus concentration yields a straight line. And by creating a series of standards it is possible to determine the amount of a substance in a sample.

Scope

This standard operational procedure applies to a basic laboratory spectrophotometer which measures absorbance in the visible range (280–760 nm). Shorter wavelengths (UV or ultraviolet) or longer wavelengths (IR or infrared) require other instruments.

Reagents

Water distilled

Equipment

- Spectrophotometer
- Cuvettes
- Water bottle
- Cleaning tissues (Lint-free wipes)

Procedure

In order to measure the absorbance of a particular substance in a reaction mixture it is necessary to first zero out the spectrophotometer such that only the substance of interest is measured. This is done with a blank or a cuvette which contains all the carrier solvents except the substance of interest. *NOTE:* A separate blank is required for every unique reaction mixture.

Operation of the Spectrophotometer

Set up

1. Turn on the instrument and allow it warm up for 5 - 10 minutes.
2. Set wavelength using the appropriate dial.
3. Prepare a blank cuvette by adding all solvents except the substance to be measured.
4. With no sample tube in the holder, adjust the meter to read infinite absorbance (0% transmittance) using the appropriate control.
5. Using a lint-free wipe to clean the outside of the blank cuvette.
6. Raise the sample cover door and insert the cuvette and close the lid. *NOTE:* If cuvettes or tubes do not have alignment mark, use a marker to make a small vertical mark at the top of each cuvette to use for consistent alignment in the sample holder.
7. Using the appropriate control adjust to read 0.00 absorbance (100% transmittance). This is also known as setting the full scale.

Measuring absorbance or transmittance

8. Remove the blank and insert the cuvette containing the sample. Close lid.
9. Read the absorbance or transmittance as appropriate for the sample.

NOTE: When taking several measurements at the same wavelength over a short period of time there is no need to re-blank for each sample. Over longer times the unit may drift and recalibrating will be necessary. If wavelengths change then re-zeroing is required.

The spectrophotometer should be calibrated for wavelength accuracy at regular intervals (usually undertaken by a service engineer during routine maintenance). All records of such calibrations should be retained in the equipment records.

LABORATORY WATER

Summary

Purified water is obtained by distillation, ion-exchange treatment, reverse osmosis, or other suitable processes. The reliability of the laboratory water is monitored by the following test procedures.

Scope

All water used by the laboratory to prepare reagents, wash glassware, or that comes into contact with samples.

Responsibility

Laboratory Analysts perform water analysis according to SOP.
Laboratory Manager/Director assure procedures are followed.
Quality Manager specify procedures used are appropriate.

Reagents

1. Alkaline Mercuric-Potassium Iodide test solution. Dissolve 10 g of potassium iodide in 10 ml of water, and add slowly with stirring, a saturated solution of mercuric chloride until a slight red precipitation remains un-dissolved. To this mixture add an ice cold solution of 30 g of potassium hydroxide in 60 ml of water, and then add 1 ml more of the saturated solution of mercuric chloride. Dilute with water up to 200 ml. Allow the precipitate to settle, and draw off the clear liquid.
2. Potassium Permanganate, $KMnO_4$ (0.1 N) test solution. Dilute 100 ml of a 1 N solution up to 1 litre with water.
3. Silver Nitrate test solution, $AgNO_3$ (0.1 N).
4. Sulphuric Acid test solution, H_2SO_4 (2 N). Add 56 ml concentrated H_2SO_4 to approximately 500 ml water in a 1-L flask. Fill to volume with water.
5. Nitric Acid, HNO_3 (70% w/v).
6. Ammonium Hydroxide test solution NH_4OH (30 µg/100 ml). Take 0.1 ml NH_4OH and dilute to volume with water in a 100 ml volumetric flask. In 100 ml beaker, add 0.1 ml of the above solution. This beaker is the control for NH_3 in high purity water.

Equipment

- Auto pipette 1 ml
- Auto pipette 5 ml
- Dispenser
- Hotplate

Procedures

1. CHLORIDE. To 100 ml of test water add 5 drops of nitric acid and 1 ml of silver nitrate. No cloudiness should be produced. *NOTE:* Use chlorinated tap water as a control.
2. CONDUCTIVITY. Use conductivity meter to test that the water sample has a resistivity greater than 16.6 Megohm at 25 °C.

3. OXIDIZABLE SUBSTANCES. To 100 ml of test water add 10 ml of 2 N sulphuric acid, and heat to boiling. Add 0.1 ml of 0.1 N potassium permanganate, and boil for 10 minutes. The pink colour should not completely disappear.
4. AMMONIA. Add 2 ml of alkaline mercuric potassium iodide test solution to 100 ml of test water: Any yellow colour produced immediately should not be darker than the control containing 30 µg of added NH_3 in 100 ml of high purity water.

LABORATORY GLASSWARE CLEANING PROCEDURES

Summary

This standard operational procedure describes the various methods used to clean the glassware in the laboratory. Broken, chipped, cracked or etched glassware is set aside for repair by a glass blower or discarded in the waste container designated for broken glass. The attention to cleaning procedures is determined by the sensitivity and accuracy of the results required. Different methods of cleaning glassware are necessary for several reasons. Glassware used in the preparation of microbiological media must have all bacteriostatic or bactericidal material removed. Sterile disposable ware such as Petri dishes and pipettes must have a certificate of sterility from manufacturer prior to being used in the laboratory. In some cases the glassware must be cleaned according to the sensitivity of the instrumentation used in the analysis. For the trace metal analyses the glassware is cleaned with acid before each use. In others, like a mycotoxin lab, the glassware must be treated to remove any remaining toxin. Therefore, it is necessary to have several unique cleaning procedures in order to address the different needs of each laboratory and the analyses they perform.

Scope

This standard operational procedure applies to all laboratory glassware used during the production of laboratory reagents and/or the preparation of samples.

Responsibility

Laboratory Analysts clean glassware according to SOP.
Laboratory Manager/Director ensure procedures are followed.
Quality Manager specify procedures used are appropriate.

Reagents

- Laboratory Detergent (DeSCAL, Contrad NF, Dri-CONTRAD or similar)
- Nitric Acid, Trace Metal Grade
- Water, distilled/deionised
- Bleach

Equipment

- Glassware dishwasher
- Deionised water purification system
- Large capacity drying oven 75 °C
- Large capacity drying oven 110 °C

Procedures

Mycotoxin/Biosafety Glassware

1. Use acetone to remove any writing on the glassware.
2. Rinse thoroughly with tap water, ensuring there are not any particles left on the glassware that may clog the washer.
3. Load in washer so that each piece will be washed and rinsed properly.
4. Start the washer. Allow the washer to flush for approximately 3 minutes and then fill with water and add 350 ml of detergent (DRI-CONTRAD or similar) to the wash.
5. Close the washer door and press start on the control panel.
6. After the wash cycle is complete, allow the glassware to cool and put them in the appropriate oven.
7. Put glassware in the 110 °C oven and plastic ware in the 75 °C oven. Glassware that contains rubber seals (i.e. blender jars) should be dried in the 75 °C oven.

General Laboratory Glassware

1. Objects that are too large to be washed effectively in the washer, should be soaked in a 10% detergent (Contrad NF or similar) bath.
2. Use acetone to remove any writing on the glassware.
3. Rinse thoroughly with tap water, ensuring there are not any particles left on the glassware that may clog the washer.
4. Load in washer so that each piece will be washed and rinsed properly.
5. Start the washer.
6. After the wash cycle is complete, allow the glassware to cool and put them in the appropriate oven.
7. Put glassware in the 110 °C oven and plastic ware in the 75 °C oven. Glassware that contains rubber seals (i.e. blender jars) should be dried in the 75 °C oven.

Trace Metals Glassware

1. Objects that are too large to be washed effectively in the washer, should be soaked in a 10% detergent (DeSCAL or similar) bath.
2. Use acetone to remove any writing on the glassware.
3. Rinse thoroughly with tap water, ensuring there are no particles left on the glassware that may clog the washer.
4. Load in washer so that each piece will be washed and rinsed properly.
5. Start the washer.
6. Allow the washer to flush for approximately 3 minutes and then fill with water and add detergent to the wash. Close the washer door and press start on the control panel.
7. After the wash cycle is complete, allow the glassware to cool and put them in the appropriate oven.
8. Put glassware in the 110 °C oven and plastic ware in the 75 °C oven. Glassware that contains rubber seals (i.e. blender jars) should be dried in the 75 °C oven.

Cleaning Procedures Using Baths/Tubs **(Nitric Acid Bath, 10%)**

All glassware used in mineral methods must be cleaned using a 10% HNO_3 solution bath. Only Trace Metal grade HNO_3 should be used when making the acid bath. *SAFETY NOTE:* Put on protective equipment before working with the 10% HNO_3 solution bath. You should have on protective gloves, a face shield and an apron or lab coat.

1. Use acetone to remove any writing on the glassware.
2. Rinse the glassware as well as possible to avoid contamination of the 10% HNO_3 solution bath.
3. Make sure the glassware is completely submerged in the bath and allow it to soak for at least two hours.
4. Carefully remove the glassware from the bath, making sure not to splash the acidic solution on you.
5. Rinse thoroughly with deionised water.
6. Put glassware in the 110 °C oven and plastic ware in the 75 °C oven. Glassware that contains rubber seals (i.e. blender jars) should be dried in the 75 °C oven.

Detergent Bath, 10% DeSCAL or Contrad NF or similar detergents

All glassware that is too large to be washed effectively in the dishwasher should be cleaned using the detergent bath. *SAFETY NOTE:* Put on protective equipment before working with the 10% detergent bath. You should have on protective gloves and an apron or lab coat.

1. Use acetone to remove any writing on the glassware.
2. Rinse the glassware as well as possible to avoid contamination of the detergent bath.
3. Make sure the glassware is completely submerged in the bath and allow it to soak for at least two hours.
4. Carefully remove the glassware from the bath, making sure not to splash the solution on you.
5. Rinse thoroughly with tap water followed by deionised water.
6. Put glassware in the 110 °C oven and plastic ware in the 75 °C oven. Glassware that contains rubber seals (i.e. blender jars) should be dried in the 75 °C oven.

Cleaning Glass Pipettes

The pipette baths are made up of a 10% detergent (DeSCAL or similar) solution. To make a fresh pipette bath, add 13.5 litres of water to the bath followed by 1.5 L of detergent

1. Make sure the glassware is completely submerged in the bath and allow it to soak for at least two hours.
2. Carefully remove the glassware from the bath, making sure not to splash the solution on you.
3. Use the pipette washer to rinse the pipettes at least three times with deionised water.
4. Put the pipettes in the 110 °C oven to dry.

Special Cleaning Procedure

Fritted glass Gooch crucibles (used for some gravimetric methods):

1. Rinse with tap water.
2. Soak in 1:7 Ammonium Hydroxide solution for at least two hours.
3. Attach Gooch crucibles to vacuum stand.
4. Rinse three times with distilled water.
5. Air dry at room temperature.

Volumetric Flasks. Be careful not to use excessive heat as this may invalidate the calibrated volume, checks with weighed water should be undertaken if excessive heat has been used.

LABORATORY SAFETY

Summary

As an employee you have a responsibility to do your job well and safely. A priority of the laboratory is to offer you a workplace free from recognizable and avoidable hazards to your health and safety.

Safety, however, cannot be mandated, nor is it something that can be given to an employee. Rather, you must make a conscious effort to help ensure safe conditions for yourself and for other workers in the area. This requires an understanding of potential hazards of the job and knowledge of the policies and regulations in dealing with those hazards.

There are unavoidable sources of risk in any work environment. The laboratory is certainly no exception. To keep the hazards from causing injuries, each individual must know how to use the tools and equipment safely and be informed of what to do in case of a fire, injury, or other emergency. However, information is not enough. Safety on the job is an attitude as much as it is knowledge.

It means:

- Recognising that accidents are not limited to those people who do not know how to prevent them. It is often the seasoned veteran, the person who "knows better," who becomes a victim by allowing familiarity to dull the edge of caution.
- Maintaining constant awareness. It involves your personal commitment to do every job safely.
- Alerting other workers to danger if they are not following the safety procedures.
- Notifying one's supervisor immediately of defective emergency equipment or other potential dangers.
- Participating on safety committees, assisting in making safety inspections, and ensuring that all safety practices are carried out every time.

Safety is an essential part of every job you perform, but no work is so important that there is not time to do it safely and correctly!

Scope

All laboratory areas including sample receiving, sample storage, and analytical laboratories.

Procedures-General Safety Rules

- There is no eating or drinking in the lab. Food is not permitted to be stored in any laboratory refrigerators, and the ice machine is not to be used for food or drink.

- Lab doors are to be kept closed in order to comply with local Fire regulations and increase air balance within the lab.
- Smoking is not allowed.
- Visitors not on official business are to be discouraged from coming into the lab. All visitors must sign in at reception, obtain personal protective equipment and be accompanied by an employee while in the lab.
- Upon entering the lab, all visitors will be required to wear personal protective equipment (safety glasses if their own eyewear is not appropriate). If they refuse, they are to be politely requested to leave; explaining the laboratory cannot assume the risk of an injury.
- Children under working age will not be allowed in the lab without the approval of the manager or director.
- Keep work area clean and uncluttered.
- All personnel must be aware of the hazards involved in the handling of any chemical/substance or performance of any procedure that they might be required to perform. It is the responsibility of each individual to become properly and completely informed of all chemicals/substances used in their area and their associated hazards.
- Before leaving an assigned laboratory work area for any reason, ensure no risk is being created by leaving the equipment/procedure unattended.
- Never perform hazardous lab procedures unless someone else is present in the building. Consult your supervisor as to what constitutes a hazardous procedure.
- Inform appropriate personnel and the supervisor in your work area when a non-routine hazardous condition may exist.
- Post appropriate warning signs in areas where non-routine hazardous conditions may exist.
- Memorize where fire extinguishers, showers, and eyewashes, chemical spill kits and first aid kits are located, as well as their proper use, so that they can be utilized rapidly and efficiently in an emergency.
- Check showers on a regular basis for efficacy and hygiene
- Lone working should be avoided

Contact your supervisor if:

- There is no specific rule governing the safe handling of a chemical or a procedure, or a rule does not seem to apply to a specific case. Consult with your supervisor or a safety advisor before proceeding.
- Any doubt exists as to the proper precautions to be taken, or if you do not understand directions or equipment. Remember that it is your health, your safety and the safety of your colleagues that is the primary concern.
- Before performing a procedure for the first time. Read the instructions carefully and discuss it with your supervisor. This will reduce confusion as to how the procedure may be safely performed.
- Only those fully trained (with up to date training files) can train others on the use of critical equipment

How to use a fire extinguisher

If a small fire occurs in your laboratory that you are confident to tackle, follow the procedures below to control it:

1. Notify other personnel that a fire exists.
2. Locate the nearest fire extinguisher suitable for the type of fire.*
3. Remove the safety pin from the handle (break the plastic tag, which attaches the pin to the extinguisher).
4. Point the nozzle of the fire extinguisher at the base of the flame, then squeeze the release arm.
5. Use the fire extinguisher in this way until the flame is out, sweeping the area to fully cover the area.
6. Ensure that the fire is extinguished, back away from the area, and then notify your supervisor.
7. Be sure that the fire extinguisher is recharged after each use, by notifying the Environmental Health and Safety Department.

* Do not use a water or foam extinguisher on electrical equipment.
Do not use a water based extinguisher on oil or solvents.
CO_2 extinguishers may be used on most types of fire.

IF A LARGE FIRE OCCURS DO NOT ATTEMPT TO TACKLE YOURSELF, ACTIVATE THE ALARM, CALL THE FIRE SERVICE AND FOLLOW EVACUATION PROCEDURES:

Procedures-Personal Safety Rules

- Appropriate eye protection must be worn at all times while in the laboratory, prep lab and the hallways in the lab. This consists of at least prescription glasses or safety glasses. Prescription or safety glasses are inadequate protection for some procedures. Lab goggles and/or face shields should be worn while performing or in the vicinity of these procedures:
  1. Waste disposal and clean-up
  2. Working with acid baths, and washing glassware which contain/contained hazardous substances.
  3. Perchloric acid digests.
- The only exceptions to wearing safety glasses in the laboratory is when looking into the microscope, setting up the PCR reactions or loading the agarose gels.
- Wearing lab coats is mandatory in most laboratories.
- Ear plugs or hearing protectors must be worn in any room when there is a high noise level.
- Wear the appropriate protective gloves when the health hazard is greater than 2. The health hazard is the number in the blue section on the NFPA diamond. Wash your hands after removing protective gloves.
- Long hair should not be allowed to interfere when working with chemicals or mechanical devices and should be tied back at all times

- Sandals cut out or toeless shoes, etc. may not be worn by laboratory or sample preparation personnel while working.
- Guidelines for specific chemical hazards may be found under SPECIAL CASES section of this manual.
- Remove gloves prior to touching door knobs, telephones, light switches, etc. to avoid contamination of common surfaces.

Procedure – Chemicals

Storage

- Store acids and bases in separate locations, near floor level and in labelled cabinets.
- Isolate perchloric acid from organic materials and from sulphuric acid. Do not store perchloric acid on a wooden shelf.
- Segregate highly toxic chemicals and carcinogens from all other chemicals.
- Do not store peroxide forming chemicals (e.g. ethyl ether, dioxane) for more than twelve months or beyond the date recommended by the manufacturer.
- Refrigerate flammables in an explosion/spark-proof refrigerator.
- Glass chemical containers should never be stored on the floor
- Ensure a containment tray is under the bottles in case of spillage.

Return chemicals, supplies, and associated equipment to their proper place after use.

If a container is to be used for waste, remove, cover or deface the original label. Then label it clearly with a marker or label so that its contents will be obvious. When chemical bottle is empty also remove or deface the label.

Electrically ground all metal solvent containers prior to transfer of any solvent.

Liquid chemical bottles should be secured when transporting between laboratories.

Never pour toxic or water insoluble flammable solvents down the sink.

To prepare ethyl ether containers for disposal, be sure to add ferrous sulphate to eliminate the danger of explosion due to the formation of peroxides.

When mixing solutions, ALWAYS add the concentrated liquid to the dilute liquid. Always add acid to water.

Organics should not be evaporated in the hood. Use a waste container to dispose of them. Organic waste containers must be capped when not in use.

Common Laboratory Chemical Hazards

Material safety data sheets (MSDS) should be easily retrievable for all chemicals/substances stored in the laboratory.

Acetic acid. Dangerous when in contact with chromic acid, sodium peroxide or nitric acid; should be stored away from oxidizing materials.

Acetone. A volatile liquid. Gives off vapours which form flammable and explosive mixtures with air; do not mix with chloroform.

Ammonia (anhydrous). Irritant, extremely caustic liquid and gas and reacts violently with strong oxidizing agents. Isolate from other chemicals, particularly chlorine and strong acids.

Ethyl ether. A highly volatile liquid. Spontaneously explosive. Peroxides sometimes form on standing. Isolate and keep away from any source of ignition.

Formaldehyde. Exposure to high concentrations may cause skin irritation and inflammation of mucous membranes, eyes, and respiratory tract.

Formic acid. Corrosive and has caustic effects on the skin. Flammable, may form explosive mixtures with air.

Hydrochloric acid. Aqueous solution is corrosive and irritating. Fumes are corrosive and irritating to mucous membranes. Hydrogen is evolved upon contact with metals. Keep away from oxidizing agents.

Hydrocyanic acid. Poisonous, inhalation may cause loss of consciousness and death. Avoid contact with skin; forms explosive mixtures with air. Keep away from any source of heat.

Hydrofluoric acid. Acid and vapours highly toxic and irritating to skin, eyes and respiratory tract. Reacts with glass; isolate and ventilate upon storage.

Hydrogen peroxide. Prolonged exposure to vapour irritating to eyes and lungs. Causes skin irritation. May decompose violently if contaminated with copper, iron, or chromium; store in cool place away from combustible materials.

Methanol. Flammable and toxic. Avoid contact with eyes and breathing in vapours.

Nitric acid. Corrosive and causes severe burns on contact with skin. Reacts vigorously with aniline, hydrogen sulphide, flammable solvents, hydrazine and metal powders.

Oxalic acid. Forms explosive compound with silver and mercury. Oxalates are toxic. Avoid skin contact.

Potassium cyanide. Highly poisonous when ingested. Evolves hydrocyanic acid gas on contact with acids or moisture.

Potassium hydroxide. Generates heat on contact with water; store in dry place.

Salicylic acid. Combustible solid; store in dry place.

Sodium hydroxide. In the same class as potassium hydroxide: Isolate from heat and water. When in contact with water liberates excess heat and irritating gases.

Sulphuric acid. Corrosive, dangerous fumes if there is fire. May ignite on contact with combustible materials; corrodes metal. Isolate from combustible materials. Always add acid to water.

Other Special Reagents

Ethers. Extremely volatile and flammable; vapours are heavier than air and may result in flashback; form potentially explosive peroxides upon exposure to air and light; store in a well-ventilated area; do **not** store in a refrigerator unless it is an explosion-proof type.

Perchloric acid. Reacts violently with organics; safe use requires special fume hood with water wash down system; must be stored to prohibit contact with organic materials (e.g. wood).

Procedures-Chemical Spills. How a spill is contained and cleaned up is of critical importance. Improper containment or clean up technique may make the situation worse than if left alone. This section gives some specific guidelines on containing and cleaning up spills.

There are six classes of hazards associated with spills, but many of these can be treated using the same technique. The general hazard classes are:

Flammables/Organics	Reactive (to either air or water)
Acids	Toxics
Bases	Biologicals

These classes have both solid and liquid chemicals within them.

Containment

Ensure that Mercury and acid spill kits are available at point of use.

Simply put, containment is preventing a spill from growing in size and effects (migration). Depending on the location of the spill and chemical(s) involved, containment can be as simple as closing a door (to restrict access and prevent dust generation), or it can necessitate the use of dykes, drain plugs, and other devices. Containment is the first step in cleaning up the spill, and prevents the spill from migrating while first responders tend to injuries and exposures to personnel. Always USE PROPER PERSONAL PROTECTION before addressing any spill. Prepare and protect yourself from the worst scenario possible.

Solid chemical spills are usually self-contained. Take measures to prevent the spilled solid chemical from spreading or generating dust. Restrict traffic around the site, and forbid any mopping/sweeping activity in the spill area until after the spill has been cleaned up.

Liquid chemical spills require dyking to prevent spreading. It is preferable to contain a liquid spill towards the middle of the room, if this is possible. This is particularly true of acids and organics, which will easily migrate under and through baseboards, cabinets, and furniture, and thus compound the spill problem greatly. An enclosing dyke may be made using several different things: long universal sorbent socks, spill pillows, sand, clay litter or vermiculite – anything that will prevent the liquid from spreading AND will not react with the spilled liquid. Drain mats are required to prevent the spill from migrating into plumbing, sewers, and waterways, if a drain exists within the spill site.

Use common sense in preventing spill migration; for instance, every floor has some slope to it however minor, and spilled liquids always migrate down that slope. Drains should be blocked first, in case the spill gets past the dyke. Proper containment will provide a much easier clean-up of the spill. In some cases, it may be necessary to dyke and contain a spill that the spill response team has been called in to handle.

Always ensure personal safety first, evacuate area and beware of fumes.

Clean up

Cleaning up a spill will usually follow the general guidelines set forth below. Unusual clean up procedures will likely need the presence of the trained spill response team. In any event, the USE OF PROPER PERSONAL PROTECTION AND CLEAN UP EQUIPMENT is required for cleaning any spill in a safe, efficient manner.

Reference should be made to local risk assessments

Dry/crystalline/powdered solid chemical spills:

1. Using a plastic broom and dustpan, scoop as much of the spilled chemical as possible. Be careful to use the brush as a stop to help in using the pan to scoop the chemical up, and not as a sweep to push the chemical onto the pan. The latter

generates dust in the cleaning process. If necessary, use a fine mist of water to suppress dustiness, but ONLY if it is safe to do so.
2. Dispose of the spilled chemical, and all disposable items used to clean up the spill, into a spill bag. Tie it off, and attach a waste disposal tag.

Liquid chemical spills (including Organics/Flammables, Acids, and Bases):

1. After containment (described previously), begin placing spill pillows along the inside of the dike, working toward the centre of the spill area. Replace soaked pillows with fresh ones as needed. DO NOT try to neutralize the spilled chemical (or spill pillows) when working with an acid/base spill. This can potentially cause greater problems (by poison gas production or unnecessary heat of reaction generation).
2. Use the emergency exhaust ventilation during the clean up to clear away accumulated vapours. Make sure fume hoods are fully open.
3. After removal of the spilled liquid, remove all traces of the chemical from the spill area. For acid or base spills, neutralize the pH of the affected area. Mop the area with a vinegar solution (for bases) or a baking soda solution (for acids). Check the pH of the area with litmus paper (if available) after mopping; the pH of the area should be neutral (pH 5–9).
4. CAUTION: If the spill involved a solution containing heavy metals (cadmium, arsenic, selenium, lead, and mercury), the wash water must be captured, and disposed of appropriately.
5. For an organic/flammable solvent spill, rinse the area with soapy water, and soak up with spill pillows. Depending on the chemical, access to the spill site may need to be restricted while any residue evaporates.
6. Dispose of the used spill pillows, and all disposable items used to clean up the spill, into a spill bag. Tie it off, and attach a waste disposal tag.

The following chemical types are classified as *special cases*. Spill handling involving these chemicals requires the use of special personal protection, clean up equipment/materials and/or special techniques. Cleaning these spills (exception: biological spills) will require notification of the safety officer, either for requesting spill response, or for consultation concerning our own response.

Mercury and its compounds have a large potential for health and environmental danger because they form vapours easily and are readily absorbed through the skin. In spill incidents, containment and clean-up must be handled with extreme care. Small, contained spills may be handled using commercially available mercury clean up kits. Larger or unconfined spills (including those of pure standards), or mercury found in sink traps, must be cleaned up by EHSD. Sulphur dust should not be used in cleaning a mercury spill; while it does capture the mercury well, the dust will only compound efforts to restore the spill site to normal occupancy/activity.

Chlorinated organic solvents pose similar dangers as those listed above, for the same reasons: they form vapours easily and are readily absorbed through the skin. Chloroform is the most common chemical of this class, affecting both the nervous and respiratory systems, with significant inhalation of its vapours causing loss of consciousness. Special care must be exercised when approaching and handling a spill involving this chemical.

Carbon Disulphide and Ammonium Hydroxide all have vapours that easily migrate to other areas to hinder normal occupancy and activity. These compounds are known to cause severe respiratory and/or optical damage, even in small amounts. Persons exposed to significant concentrations of vapours from these chemicals require prompt medical attention. A spill involving any of these chemicals requires special personal protection and handling for a safe response, containment and clean up.

Carbon Disulphide is extremely flammable, which makes elimination of all spark and ignition sources first priority. Its low boiling point (46 °C) requires a very rapid response time. Personal protection must include full-face protection (vapours cause eye irritation), and all clean up equipment must be non-metallic. Persons exposed to carbon disulphide require medical attention immediately, as prolonged inhalation of vapours may cause damage to both the central and peripheral nervous systems, as well as to the liver and kidneys.

While not included in the list of *hazardous chemicals*, **ammonium hydroxide** is a very potent and noxious base that can overwhelm a respirator cartridge in a spill of a litre or more. It causes severe burning of the nasal and respiratory linings, and with prolonged exposure can cause respiratory collapse. Self-contained breathing equipment should be used for containing and cleaning up spills larger than a 4-litre bottle.

Biological Spills. This category of spill will be one of three types. The first is that of bloodshed from a severe wound which has occurred in the workplace. The second is a punctured/torn autoclave bag dripping waste (prior to autoclaving). The third involves the spilling of bacterial suspensions. Each of these three scenarios are potentially dangerous but all are handled in the same way: wearing personal protective clothing wipe the area clean, and follow up with a sodium hypochlorite (5%) or bleach (10%) solution. This will destroy any biohazardous agents that could spread from the spill site. Take extreme care when dealing with any blood spills and treat as HIV or Hepatitis positive as a precaution.

Mycotoxins. All of the mycotoxin samples and standards handled in the lab are highly carcinogenic, targeting the liver, brain, and central nervous system. All are inhalation and ingestion hazards. Gloves must be worn whenever handling mycotoxin samples. Any spill involving mycotoxins requires special cleaning steps and acute attention to personal safety. The spill site must be decontaminated with a sodium hypochlorite (5%) or bleach (10%) solution for 30 minutes then cleaned again with acetone. Press the Emergency Ventilation button and evacuate the room until all fumes have dissipated.

GENERAL PROCEDURES – CORRECT USE OF LABORATORY EQUIPMENT

1. Autoclaves
2. Electrophoresis
3. Microbiological BSL2 (Containment Level 2) safety
4. Mycotoxin BSL2 (Containment Level 2) safety
5. Vacuum/pressure
6. Electrical equipment
7. Lifting
8. Flammable liquids
9. Gas cylinders
10. Glassware

11. Sharps
12. Fume hoods
13. Special precautions for perchloric hoods

1. Autoclaves

- Autoclaves operate under steam pressure of 18–20 psi (0.12–0.14 MPa). The resulting temperatures are 121 °C or more. Even if familiar with the use of the equipment, new personnel should ask for assistance with operation, packaging, loading and labelling procedures before using.
- For the autoclave process to be effective in achieving sterilisation, sufficient temperature, time, and direct steam contact are essential. Air must be completely removed from the steriliser chamber and from the materials to allow steam penetration so that the material being autoclaved will be at treatment temperature for sufficient time to achieve sterilisation.
- Jacket pressures will remain at 18–20 psi (0.12–0.14 MPa) and 121 °C. Chamber pressure will reach 18–20 psi (0.12–0.14 MPa) and 121 °C during sterilisation.
- Super-heated liquids quite frequently boil over when slightly shaken and may result in burns. Always use protection recommended for handling hot materials.
- Use the appropriate cycle (gravity or liquid) for the time necessary for the item being autoclaved.
- If you have any questions about the use of the equipment, talk with your supervisor.

Packaging

- Use containers labelled "Biohazard" for microbiological waste.
- Do not seal containers or bags tightly.
- Do not put sharp objects such as broken glassware into an autoclave bag.
- You must place a strip of autoclave tape (displays a colour change when autoclaved at 121 °C) on any item being autoclaved or include a suitable indicator (e.g. Browne's Tube) to indicate that the target temperature has been met.

Loading

- Place containers that may boil over or leak (agar plates, etc.) inside an autoclavable pan.
- Never place items in direct contact with the bottom of the autoclave.
- Do not overload; leave sufficient room for thorough steam circulation.
- Make sure that the plug screen in the bottom of the autoclave is clean.

Documentation

- Document the treatment of each load of autoclaved waste for the date of treatment, amount of waste treated, method of treatment, date and initial.

2. Electrophoresis

Electrophoresis equipment may be a major source of electrical hazard in the laboratory. The presence of high voltage and conductive fluid in this apparatus presents a potentially lethal combination.

Many people are unaware of the hazards associated with this apparatus; even a standard electrophoresis operating at 100 volts can deliver a lethal shock at 25 milliamps. In addition, even a slight leak in the device tank can result in a serious shock.

Protect yourself from the hazards of electrophoresis and electrical shock by taking these precautions:

- Use physical barriers to prevent inadvertent contact with the apparatus.
- Use electrical interlocks.
- Frequently check the physical integrity of the electrophoresis equipment.
- Use warning signs to alert others of the potential electrical leads.
- Use only insulated lead connectors.
- Turn the power off before connecting the electrical leads.
- Connect one lead at a time using one hand only.
- Ensure that your hands are dry when connecting the leads.
- Keep the apparatus away from water and water sources.
- Turn the power off before opening the lid or reaching into the chamber.
- Do not disable safety devices.
- Follow the equipment operating instructions.

3. Microbiological BSL2 (Containment Level 2) Safety

- Access to the microbiological laboratory is limited to individuals who are working in the lab. **The laboratory supervisor must ensure that personnel working in BSL2 laboratory receive appropriate training regarding their duties, the necessary precautions to prevent exposures, and exposure evaluation procedures. The training must be documented in the individual's training file.**
- Persons who are at increased risk of acquiring infection (immune compromised), or for whom infection may have serious consequences (such as pregnant women), may not be allowed in the microbiological laboratory or have the work they perform restricted. The Laboratory Director has the final responsibility for assessing each circumstance and determining who may enter or work in the microbiological laboratory.
- Laboratory personnel will receive appropriate immunizations or test for the agents handled or potentially present in the microbiological laboratory.
- When appropriate, considering the agent(s) handled, a baseline serum sample for microbiological laboratory staff and other at-risk personnel will be collected and stored.
- Personnel must receive annual lab specific safety training.
- Personnel must receive additional training when procedural or policy changes occur.
- Personal health status may impact on an individual's susceptibility to infection, ability to receive immunizations or prophylactic interventions. Therefore, all laboratory personnel and particularly women of child-bearing age should be provided with information regarding immune competence and conditions that may predispose them to infection. Individuals having these conditions should be encouraged to contact the institution's healthcare provider for appropriate counselling and guidance.
- Entry Procedure

  - Wear clean lab coat whenever you enter the lab or put one on immediately upon entry.
  - Put on protective gloves before handling potentially infectious materials.
- Individuals not listed on the authorized personnel list, located on the outside of the door, can only be admitted by the Director or the Microbiological Laboratory Supervisor and they must be accompanied by an authorized individual at all times.
- Perform all procedures carefully to minimize the creation of splashes or aerosols.
- Decontaminate all work surfaces on the completion of work or at the end of the day and after any spill or splash of viable material with disinfectants that are effective against the agents of concern.
- A high degree of precaution must always be taken with any contaminated sharp item, including needles, syringes, slides, capillary tubes and scalpels.
- Plastic ware should be substituted for glassware whenever possible.
- Only needle-locking syringes or disposable syringe-needle units are used for injection or aspiration of infectious materials.
- Used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes or otherwise manipulated by hand before disposal; rather, they must be carefully placed in conveniently located puncture-resistant, autoclavable containers used for sharps disposal.
- Broken glassware must not be handled directly by hand, but must be removed by mechanical means such as a brush and dustpan, tongs, or forceps. Containers of contaminated needles, sharp equipment, and broken glass are decontaminated before disposal, according to local regulations.
- Cultures, tissues, specimens of body fluids, or potentially infectious wastes are placed in a container with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping.
- Contaminated equipment must be decontaminated according to applicable regulations before it is sent for repair or maintenance or packaged for transportation.. Decontamination should be documented.
- Immediately report all spills and accidents that result in overt exposure to infectious materials to the Laboratory Director and Safety Officer.
- Provide medical evaluations, surveillance, and treatment as appropriate and maintain written records.
- Immediately report all spills and accidents that result in overt exposure to organisms that contain recombinant DNA.
- An insect and rodent control program must be in effect.
- A properly maintained biological safety cabinet must be used whenever conducting procedures with a potential for creating infectious aerosols or splashes.
- Cloth chairs are not permitted in the microbiological laboratory.
- Face protection (goggles, mask, face shield or other splatter guards) must be used for anticipated splashes or sprays of infectious or other hazardous material to the face when microorganisms must be manipulated outside the biosafety cabinet.
- Gloves must always be worn whenever handling potentially infectious materials, contaminated surfaces or equipment.

- Dispose of gloves whenever they are overtly contaminated, when work with infectious material is completed, or when the integrity of the glove is compromised. Disposable gloves are not to be washed, reused, or used for touching "clean" surfaces (keyboards, telephones, etc.).
- Protective laboratory coats must be worn while in the microbiological laboratory.
- Dirty lab coats should be sterilized and washed as appropriate. If you are planning to wear your lab coat again, hang it up in the microbiological laboratory on the coat rack. Lab coats should never be worn outside the microbiological laboratory once they are worn inside the microbiological laboratory.
- Lab coats will be put in the washer by BSL2 personnel.
- Do not make or answer phone calls while in the microbiological laboratory.
- Individuals wash their hands after they handle viable materials, after removing gloves and **before** leaving the microbiological laboratory.
- Exit Procedure
  - Remove gloves.
  - Remove lab coat.
  - Hang up lab coat or send for sterilizing and washing.
  - Wash hands.
- Custodial duties will be performed by BSL2 personnel under the direction of the microbiological laboratory supervisor.
- Materials to be decontaminated outside of the immediate microbiological laboratory are placed in a durable, leak proof, autoclavable biological waste bag and closed for transport from the laboratory.
- All containers and vials will be wiped down with 10% bleach, 5% sodium hypochlorite or 70% alcohol before exiting the BSL2 lab for storage. *NOTE:* Avoid using alcohol on vials to prevent the ID markings from being removed.

4. Mycotoxin BSL2 (Containment Level 2) Safety

- Access to the mycotoxin laboratory is limited to individuals who are working in the lab.
- Persons who are at increased medical risk due to toxin exposure are not allowed in the mycotoxin laboratory. The Director has the final responsibility for assessing each circumstance and determining who may enter or work in the mycotoxin laboratory.
- Laboratory personnel will have access to appropriate medical tests for the agents handled or potentially present in the mycotoxin laboratory.
- When appropriate, considering the agent(s) handled, a baseline serum sample for mycotoxin laboratory staff and other at-risk personnel will be collected and stored.
- All individuals working in the mycotoxin lab will be appropriately trained on the potential hazards associated with the work involved, the necessary precautions to prevent exposures, and the exposure evaluation procedures.
- Entry Procedure
  - Wear clean lab coat whenever you enter the lab or put one on immediately upon entry.
  - Put on protective gloves before handling potentially toxic materials.

- Individuals not listed on the authorised personnel list, located on the outside of the door, can only be admitted by the Director or the Mycotoxin Laboratory Supervisor and/or they must be accompanied by an authorised individual at all times.
- Toxin samples and standards can only leave the Toxin BSL2 lab in HPLC vials to be run on an analytical instrument, all other toxin work must be done in the BSL2 Toxin lab.
- All toxin samples, extracts and standards must be stored in the BSL2 Toxin lab.
- Perform all procedures carefully to minimise the creation of splashes or aerosols.
- Decontaminate all work surfaces on the completion of work or at the end of the day and after any spill or splash of viable material with deactivation agents that are effective against the toxins of concern. For most mycotoxins, this will be 10% bleach followed by 5% acetone.
- A high degree of precaution must always be taken with any contaminated sharp item, including broken glass, needles, syringes, slides, capillary tubes and scalpels.
- Plastic ware should be substituted for glassware whenever possible.
- Broken glassware must not be handled directly by hand, but must be removed by mechanical means such as a brush and dustpan, tongs, or forceps. Containers of contaminated needles, sharp equipment, and broken glass are decontaminated before disposal, according to any local, state, or federal regulations.
- Potentially toxic wastes are placed in a container with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping.
- Contaminated equipment must be decontaminated according to any local regulations before it is sent for repair or maintenance or packaged for transportation in accordance with applicable regulations, before removal from the facility.
- Immediately report all spills and accidents that result in overt exposures to toxic materials to the Laboratory Director and Safety Officer.
- Provide medical evaluations, surveillance, and treatment as appropriate and maintain written records.
- An insect and rodent control program must be in effect.
- Cloth chairs are not permitted in the mycotoxin laboratory.
- Gloves must always be worn whenever handling potentially toxic materials, contaminated surfaces or equipment.
- Dispose of gloves whenever they are overtly contaminated, when work with toxic material is completed, or when the integrity of the glove is compromised. Disposable gloves are not to be washed, reused, or used for touching "clean" surfaces (keyboards, telephones, etc.).
- Protective laboratory coats must be worn while in the mycotoxin laboratory.
- Put dirty lab coats in a hamper (lined with a hot-water soluble laundry bag) located in the mycotoxin laboratory. If you are planning to wear your lab coat again, hang it up in the mycotoxin laboratory on the coat rack. Lab coats should never be worn outside the mycotoxin laboratory once they are worn inside the mycotoxin laboratory.
- Lab coats will be put in the washer by BSL2 personnel, sterilise first if necessary.
- Do not make or answer phone calls while in the mycotoxin laboratory.
- Individuals wash their hands after they handle toxic materials, after removing gloves and before leaving the mycotoxin laboratory.

- Exit Procedure
  - Remove gloves
  - Remove lab coat
  - Hang up lab coat or put it in the hamper (located within the mycotoxin lab).
  - Wash hands
- Custodial duties will be performed by BSL2 personnel under the direction of the mycotoxin supervisor.
- All glassware and plastic ware that needs to be sent to the washroom will be decontaminated with bleach prior to leaving the mycotoxin laboratory.

5. Vacuum/Pressure

- Switch off vacuum when not in use.
- Use only vacuum tubing for connections to vacuum equipment.
- Apply vacuum only to glassware made specifically for that purpose, and wrap flasks with flexible tape (duct, electrical, etc.) prior to applying suction.
- Relieve vacuum slowly in all parts of system before opening apparatus.
- Inspect all vacuum equipment for flaws prior to use. Discard all chipped, cracked or broken vacuum glassware.

6. Electrical Equipment

- Do not use electrical equipment if not in good working order. Check for frayed or damaged power cords or broken control switches.
- Local electrical safety testing to be undertaken at specified regular intervals (generally once a year)
- Avoid all contact with water when using electrical equipment.
- Use grounded outlets with circuit breakers only. No extension cords.
- Ensure that instruments used in series are of the same voltage.
- Ensure that main power switch is "OFF" and instrument unplugged before servicing, if possible.
- Never by-pass any safety device.
- Do not use electrical equipment such as mixers or hot plates around flammable solvents.
- Use only carbon dioxide or dry powder extinguishers in case of fire in or near electrical equipment.
- In case of power outage, turn off or unplug equipment to prevent damage; if fumes are present close appropriate hood and doors. Evacuate if necessary.
- Do not use any electrical appliance or piece of equipment that will exceed the rated amperage of the intended circuit. Remember that any other appliance on that circuit will also need to be considered when calculating the total number of amps to be drawn. If you are uncertain about the rated amps for a given circuit, request your supervisor to ask for assistance from appropriate maintenance staff.
- If, during the course of an experiment, a circuit breaker trips, turn off all electrical appliances on that circuit and notify your supervisor.

- When using multiple outlet plugs for electrical outlets, use caution not to exceed the rated amperage of the circuit. The device should have its own circuit breaker. If you reach a point of an insufficient number of outlets, it is time to have the circuit load evaluated.

7. Lifting

- Physical differences make it impractical to set up safe lifting limits for all workers. Height and weight do not necessarily indicate lifting ability.
- For lifting an object, observe the following guidelines:
  - Inspect the object to be lifted.
  - Look and know where you are going.
  - Wear necessary protective equipment, such as gloves, aprons and safety shoes.
  - If a load is too heavy or bulky for you to handle alone, get help.
  - On a job that requires two or more people, work together. Assign one individual to call signals.
  - Use correct lifting methods:
  - Make a preliminary judgment before lifting to be sure you can handle it.
  - Set your feet solidly, one foot slightly ahead of the other.
  - Crouch as close to the load as possible.
  - Keep the back straight.
  - Take a firm grip on the object using the palms of the hands.
  - Lift with your legs and your forearms to prevent back strain.
  - Keep a firm grip on the object as it is being moved.
  - Before you put a load down, make sure your fingers and toes are clear.
  - When you must change direction, do not twist the body; use your feet to turn the whole body.
- If available use a lifting belt for heavy objects.
- Appropriate training should be provided in manual handling procedures.

8. Flammable Liquids

- **Material safety data sheets (MSDS) should be easily retrievable for all chemicals/substances stored in the laboratory.**
- All solvents should be handled carefully, even though they may be relatively inactive from a chemical point of view. Some of the commonly used solvents are volatile and are harmful when relatively small amounts are inhaled. Some are readily absorbed through the skin and most are flammable.
- Avoid generation of static sparks when transferring a flammable liquid from a drum by electrically grounding the drum.
- Store large volumes of flammable liquids in a suitable designated secure flammable storage container. Keep minimum quantities of flammable liquids in the laboratory. Use older reagents first. All flammable liquids must be stored in an approved safety container. Never return liquids to the original container.
- Keep flammable liquids away from heat, direct sunlight, and strong oxidizing agents such as chromic acid, permanganates, chlorates or perchlorates.

- Handle flammable materials in a fume hood, avoiding simultaneous use of oxidizers.
- Ensure solvent vessels are stored on drip trays.

9. Gas Cylinders

- Ensure the cap is firmly in place when storing or moving a cylinder. (This protects the valve stem from being accidentally broken off.) Move gas cylinders with an appropriate hand-truck. The protective cap must be in place before the cylinder is released from its support.
- Always support gas cylinders by straps, chains or a suitable stand to prevent them from falling over.
- Close all cylinders and bench valves when not in use.
- Ensure that the appropriate regulator is used on each gas cylinder.
- Never use a cylinder that cannot be positively identified as to its content.
- Never force a cylinder valve.
- Never use oil or grease on a regulator or tank valve.
- Do not use a regulator or tank if oil or grease is present with oxygen or other oxidant. Combustible substances in contact with an oxidant are explosive.
- Reactions requiring cylinders of toxic, flammable or reactive gases, should be run in fume hoods, and suitable racks should be provided to hold the cylinders.
- In case of fire, turn off the flammable gas, then oxidizing gas if possible!
- Do not extinguish a flame involving a highly combustible gas until the source of gas has been shut off. Otherwise it can re-ignite with an explosion.
- Remove regulator from almost empty cylinder and replace protection cap at once; using tape or tag, label the cylinder as "EMPTY".
- For cylinders used in GC/HPLC analysis moisture traps shall be used and checked regularly.

10. Glassware

- Wear heat resistant gloves or use tongs when handling glassware or equipment that has been heated.
- When glassware is ready to by cleaned, take it to the wash area, and specify any special cleaning instructions.
- Lubricate all contact surfaces and wear cut/slash resistant protective gloves when inserting glass into a stopper or tubing; insert the glass so that the applied force is away from your body.
- Do not use excessive force.
- Do not stopper glass flask containing hot condensable vapours.
- Dispose of chipped or broken glassware in the Broken Glass receptacle. All broken glassware must be immediately swept up and removed from the bench or floor and placed in a receptacle provided solely for that purpose.
- Do not place any glassware in general waste containers.
- Notify your supervisor of any significant breakage.
- Broken glassware may be placed in a rigid, sealed container (e.g. a box). Label the container "Broken Glass."

11. Sharps

- Take care not to stick yourself with a needle.
- Do not place used syringes in pans containing pipettes or other glassware that require sorting.
- Do not recap used needles.
- Dispose of needles in an approved sharps autoclavable container.
- Ensure sharps container is autoclaved before disposal.
- After the sharps container is sterilized, Plaster of Paris may be added and allowed to dry before disposal, this will immobilize the sharps and ensure that no one is accidentally stuck by them.
- Needles, blades, etc. are considered hazardous even if they are sterile, capped and in the original container.

12. Fume Hoods

- Hoods should be turned off and the sash closed when not in use. If the hood is a variable speed hood it cannot be turned off, just make sure the sash is closed when not in use.
- Equipment and other materials should be placed and all work should be performed a minimum of 15 cm (6 inches) behind the sash opening. This practice will reduce exposure of laboratory area to chemical fumes due to air turbulence.
- When the hood is in use, the sash should be kept at the optimum air flow height. A sticker affixed directly next to the sash indicates this height. The sash is your primary barrier for protection against fire and explosion that may take place in fume hoods.
- Paper and other materials should not be permitted to enter the exhaust duct of the hood. Foreign objects can be drawn into the duct work and exhaust fan, and will adversely affect the performance of the fume hood.
- Hoods are not intended for the storage of chemicals or equipment. All excess chemicals and equipment should be stored in areas designed for long term storage.
- Equipment and other materials should not be stored against the baffle area (slot at back of hood). This baffle provides a means for air movement through the fume hood. If blocked, the hood will not provide consistent air movement.
- Large equipment placed in fume hoods should be elevated a minimum of 3 - 4 cm (1½ ") above work surface to allow unrestricted air movement beneath equipment.
- While personnel are working in the fume hood, the sash should be pulled down to a level that will protect the user's face and upper chest. The only time the fume hood sash should be fully open is while setting up equipment for a procedure.
- Check that there is sufficient flow in the hood before commencing work.
- Do not rely on the fume hood exhaust to protect you from splashes or projectiles -- WEAR APPROPRIATE SAFETY EQUIPMENT DICTATED BY TYPE OF CHEMICALS AND EQUIPMENT BEING USED.
- If you question whether the hood is working properly, call the Environmental Health and Safety Department.

13. Special Precautions for Perchloric Hoods

- Persons using perchloric acid shall be thoroughly familiar with its hazards.
- Spilled perchloric acid should be thoroughly washed away with large amounts of water.
- The use of organic materials or chemicals in the hood should be avoided.
- Gas flames or oil baths should not be used within the hood.
- Goggles or face shields should be used, as well as utilization of the fume hood sash whenever possible for additional safety.

Perchloric acid fume hood wash down procedure

1. This procedure shall be performed after each use of perchloric acid.
2. Unplug all apparatus in the fume hood.
3. Close the sash.
4. Turn on the wash down spray.
5. The wash down spray must be on for a total of at least 15 minutes.

PART II

Analytical section

Analytical procedures

INTRODUCTION

Optimal animal production is mainly determined by an adequate animal nutrition from a physiological, an economical and an ecological viewpoint. In order to provide animals with the necessary nutrients to meet their requirements for maintenance, growth, pregnancy and the production of meat, milk, eggs, wool and labour, to reduce the risks for animal health and to minimize the excretions and emissions into the environment, the nutritive value of the feeds used in the diet has to be precisely known. Most animal feeds like forages and by-products of human food and bio-energy production have a variable quality, necessitating analyses per batch. As nutrient requirements are derived by using standard methods, it is extremely important that the nutrient content of the feeds is analysed with the same or similar standard methods. Moreover, the use of standard methods improves the transparency of data among labs, institutes and companies.

In the second part of this manual, commonly used methods to determine the chemical and mineral composition of feeds, as well as some important mycotoxins are described. The description of the methods is according to a fixed order of items starting with principle, scope, responsibilities, equipment, reagents, procedure, calculation, quality control, remarks, interferences and troubleshooting and references. For the equipment and reagents only the specific needs of the lab are mentioned. The procedure is described in brief, more detail can be added according to the specific facilities of each lab. The preparation of the sample is not described in the procedure, but guidelines are given in the first part of the manual. Concerning quality control, the use of a control sample in each run is extremely important to guarantee precision. In addition, duplicate analyses are recommended and strict limits to evaluate duplicates are mentioned. When a lab is not able to attain these limits, the specific intralab reproducibility should be determined and quoted when requested. In the following, the background and the relevance of the methods to analyse feeds will be discussed.

Since Henneberg and Stohmann developed the Weende scheme of analysis in 1860, the main chemical components of animal feeds are analysed using empirical methods i.e. moisture, crude protein, crude fibre, crude fat and crude ash, whereas the residual fraction of nitrogen free extractives is calculated by difference. In 1963 Van Soest developed a specific analysis scheme intending for a better characterisation of the nature of the cell walls. In Table 1 these two analysis schemes are given together with the chemical constituents they represent.

The determination of moisture followed by that of crude ash in feed, results in the content of organic matter containing the nutritive substances for the animal. Crude ash consists of insoluble ash, useless for the animal, minerals and trace elements.

Crude protein is determined as nitrogen and originates from real protein, mainly amino acids and peptides, at one side and non-protein such as ammonia, urea, nitrates, amines

at the other; the former are the direct building blocks for the formation of animal proteins; Non protein nitrogen (NPN) in the presence of energy stimulates the growth of rumen bacteria, a high quality protein source for the animal.

Crude fat mainly contains fatty acids and sometimes pigments and waxes; the former provide energy, but also building blocks for the formation of animal fat and are a source of vitamins.

The greatest fraction in most feeds is carbohydrates, which also deliver energy, and which can be extremely diverse in nature. One distinguishes structural and non-structural carbohydrates. There are several methods to characterize the structural carbohydrates. Crude fibre mainly consists of cellulose, a linear polymer of glucose and further of lignin, a polymer of phenolic acids. Neutral detergent fibre (NDF) represents total cell walls: hemicellulose, cellulose and lignin. Acid detergent fibre (ADF) is similar to crude fibre. The difference between NDF and ADF is a measure of hemicellulose, which is a branched polymer of different sugars. The difference between ADF and lignin is a measure of cellulose. Hemicellulose and cellulose are partly digestible by ruminants, but almost non digestible for monogastric animals; their digestibility mainly depends on the lignification degree. ADF may contain nitrogen as a result of protein denaturation by enzymatic browning (Maillard reaction). Pectins are also matrix polysaccharides, but they are highly digestible. Among the non-structural carbohydrates, starch, fructosans and sugars are important components.

The determination of the gross energy content of feeds gives an idea of their calorific value.

Forages, harvested in good climatic conditions, may be ensiled for feeding during less favourable periods. During ensiling, the sugars are fermented to mainly lactic and acetic acid, some ethanol and sometimes the less desirable butyric acid. Characterization of the fermentation products helps to evaluate the silage quality and may also be used to correct the DM-content of silages for loss of volatile substances during oven drying.

Besides protein and energy, the animals need minerals and trace elements. Calcium and phosphorus are two very important minerals for bone development of the animal, but also for the production of milk and eggs. Other important minerals are magnesium, sodium and potassium. Because most plants provide insufficient sodium for animal feeding and may lack adequate chloride content, salt supplementation is a critical part of a nutritionally balanced diet for animals. Trace elements are essential for enzymatic reactions in the body; their bio-availability may vary considerably. The most important ones are iron, copper, zinc, manganese, cobalt, iodine and selenium.

Because ruminants are able to digest forages and less digestible feeds like straw and bran, an *in vitro* digestibility method may be very informative for feed evaluation.

Besides nutritive substances, feeds may also contain undesirable substances. These compounds are defined as substances or products, which are present in and/or on the product intended for animal feed and which present a potential danger to animal or human health or to the environment or could adversely affect livestock production. The best known representatives are dioxins, PCB, heavy metals and mycotoxins. As typical contaminants it is impossible to fully eliminate their presence but it is important that their content in products intended for animal feed should be reduced, with due regard to the substances' acute toxicity, bioaccumulability and degradability, in order to prevent undesirable and harmful effects.

TABLE 1
Weende and Van Soest schemes for analysis of feeds and corresponding chemical constituents

<table>
<tr><th>Weende analysis</th><th colspan="3">Chemical constituents</th><th colspan="2">Van Soest analysis</th></tr>
<tr><td>Moisture</td><td colspan="3">Water</td><td colspan="2"></td></tr>
<tr><td rowspan="2">Crude protein</td><td rowspan="11">Dry matter</td><td rowspan="11">Organic matter</td><td>Protein</td><td colspan="2" rowspan="8">Neutral detergent soluble</td></tr>
<tr><td>Non-protein N</td></tr>
<tr><td rowspan="2">Crude fat</td><td>Lipids</td></tr>
<tr><td>Pigments</td></tr>
<tr><td rowspan="3">Nitrogen-free extractives</td><td>Starch</td></tr>
<tr><td>Sugars</td></tr>
<tr><td>Organic acids</td></tr>
<tr><td>Pectins</td></tr>
<tr><td>Hemicellulose</td><td></td><td rowspan="4">NDF</td></tr>
<tr><td rowspan="3">Crude fibre</td><td>Cellulose</td><td rowspan="3">ADF</td></tr>
<tr><td>Lignin</td></tr>
<tr><td>Fibre-bound N</td></tr>
<tr><td rowspan="2">Crude ash</td><td rowspan="2">Inorganic matter</td><td>Insoluble ash</td><td colspan="2">Silica</td></tr>
<tr><td>Soluble ash</td><td colspan="2"></td></tr>
</table>

ADF = acid detergent fibre
NDF = neutral detergent fibre
Note: **The values for all constituents given in Table 1 and analysed by the methods given below MUST be reported to one decimal place only.**

In almost all countries maximum levels for undesirable substances have been defined. If these are exceeded, it is forbidden to import the product and to feed. Therefore, an accurate analysis is essential to these substances. In this document methods for the most important mycotoxins (aflatoxins, fumonisins, deoxynivalenol and zearalenone) are given.

DRY MATTER

1. Principle

Dry matter is determined gravimetrically as the residue remaining after drying at 103 °C in a ventilated oven.

2. Scope

This procedure is applicable for determination of dry matter in feed ingredients, feeds and partially-dried (85% dry matter) forages with low volatile acid content. For whole grain, silage and high sugar feeds, use different procedure (see remark 9.1).

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified

4. Equipment

4.1 Aluminium dish (pan), approximately 50 mm diameter, 40 mm deep, covered.
4.2 Analytical electronic balance, accurate to 0.1 mg.
4.3 Forced-air drying oven at 103 ± 2 °C. Oven should be equipped with a shelf to allow the circulation of air. It should be operated with open vents.
4.4 Desiccator.

5. Reagents

None.

6. Procedure

6.1 Dry aluminium dish (4.1) with cover at 103 ± 2 °C for at least 2 hours.
6.2 Cover dishes and move to a desiccator (4.4).
6.3 Immediately cover desiccator and allow covered dishes to cool to room temperature. Do not allow dishes to remain in the desiccator for more than 2 hours.
6.4 Weigh dishes with cover (W1) to nearest 0.1 mg, removing one at a time from the desiccator and keeping the desiccator closed between dish removals. Use tongs to handle beakers.
6.5 Add approximately 2 g ground sample to each dish. Record weight of dish with cover and sample (W2) to the nearest 0.1 mg.
6.6 Shake dish gently to uniformly distribute the sample and expose the maximum area for drying.
6.7 Insert samples (with lids removed to the side) into a preheated oven at 103 ± 2 °C (4.3) and dry for at least 2 hours, start timing once oven has reached temperature (dry to constant weight, may need to check this for various sample types, once confirmed use that drying time).
6.8 Move samples to a desiccator (4.4), place cover on each dish, seal the desiccator and allow to cool to room temperature. Do not allow samples to remain in the desiccator for more than 2 hours.
6.9 Weigh dish with cover and dried sample (W3), recording weight to nearest 0.1 mg.

7. Calculation

Percent Dry Matter (% DM):

$$\% \text{ DM} = (W3 - W1) \times 100 / (W2 - W1)$$

where,

W1 = weight of empty dish (g),
W2 = weight of dish and sample (g), and
W3 = weight of dish and sample after drying (g).

Percent Moisture:

% Moisture = 100 – % DM (see remark 9.2)

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall not exceed 0.2% of the absolute value of dry matter. If the difference is higher than 0.2%, repeat the analysis.

9. Remarks

9.1 For determination of dry matter in grains, dry at 135 ± 2 °C for 2 hours; for silages (sample size: minimum 500 g) use 60 ± 1 °C for 24 hours and for high sugar samples (e.g. molasses, bagasse, compound feed containing more than 4% of sucrose or lactose) use 80–85 °C in a vacuum oven.

9.2 Besides moisture, other volatile compounds, such as ammonia and volatile fatty acids, disappear during drying. This should be taken into account by calculating the moisture content.

10. Interferences, troubleshooting and safety

10.1 Time and temperature must be adhered to closely.

10.2 Samples should be placed in drying oven so that air can circulate freely.

10.3 Sample dishes should not be packed excessively tightly in a desiccator. Air movement is necessary to cool sample dishes. Open the loaded desiccator very slowly after samples have cooled. A vacuum forms during cooling and abrupt opening results in turbulence which can blow samples out of uncovered containers.

10.4 Desiccator lid should be slid open for the removal of each container and closed during weighing. Leaving the lid open allows samples to absorb moisture.

10.5 Desiccant should be checked and dried periodically. Use of desiccant with colour indicator for moisture is recommended.

10.6 Use tongs to handle beakers at all times.

11. References

AOAC 930.15. 2000. *Moisture in animal feed, loss on drying at 135 °C for 2 hours*. Gaithersburg, MD, USA.

ISO 6496. 1999. *Animal feeding stuffs – Determination of moisture and other volatile matter content.* Geneva, Switzerland.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex III, A, *Official Journal of the European Union* L54/1 from 26/02/2009.

CRUDE ASH

1. Principle

Ash is determined gravimetrically as the residue after incineration at 550 °C.

2. Scope

This procedure is applicable for determination of ash in feed ingredients and feeds. This method cannot be used for mineral mixtures.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Incineration dish.
4.2 Analytical electronic balance, accurate to 0.1 mg.
4.3 Muffle Furnace, capable of maintaining temperatures to 550 ± 20 °C.
4.4 Desiccator.

5. Reagents

None.

6. Procedure

6.1 Dry incineration dish (4.1) at 103 °C for at least 2 hours, remove from oven and cool in a desiccator.
6.2 Weigh the empty dish (4.1) to the nearest 0.1 mg (W1).
6.3 Add approximately 5 g of sample to the dish and weigh to the nearest 0.1 mg (W2).
6.4 Place the dish in preheated muffle furnace at 550 ± 20 °C (4.3) for 3 hours.
6.5 Inspect visually if the residue is free from carbonaceous particles (see remark 9.1).
6.6 Transfer dish into a desiccator (4.4) and allow to cool to room temperature (approximately 45 minutes).
6.7 Weigh the dish to the nearest 0.1 mg (W3).

7. Calculation

Percent Ash (% ASH):

$$\% \text{ ASH} = (W3 - W1) \times 100 / (W2 - W1)$$

where,

W1 = weight of empty dish (g),
W2 = weight of the dish and sample (g), and
W3 = weight of dish and residue after incineration (g).

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall not exceed 0.2% in the absolute terms for contents lower than 10%, and 2% relative to the higher value for contents ≥ 10% (with a maximum difference of 0.5%-units).

9. Remarks

9.1 If the residue contains carbonaceous particles, moisten with distilled water, evaporate the water carefully to dryness at 103 ± 2 °C and incinerate for 1 hour at 550 ± 20 °C.

10. Interferences, troubleshooting and safety

10.1 Do not allow ashed sample to cool below 200 °C in the ashing oven before transferring to a desiccator because sample might absorb moisture.

10.2 Do not allow sample to remain in the desiccator at room temperature for more than 2 hours before weighing.

10.3 Take care when opening the desiccator to avoid loss of the ash.

10.4 Desiccant must be kept fresh and desiccator lid properly sealed with high vacuum grease, a silicone lubricant.

10.5 Ensure that all surfaces with which the crucibles come in contact are clean, especially desiccators where vacuum grease is used.

10.6 Re-tare balance in between each weighing.

10.7 The furnace should not be preheated. This prevents rapid ignition of sample and allows slow loss of moisture. Rapid ignition may cause flaming and rapid loss of moisture may cause splattering of sample; both of these situations could result in loss of sample.

10.8 Be aware that some samples foam out of the crucible. If this occurs, repeat the analysis using acid-washed sand and/or use a larger crucible.

HAZARDS: Use long handled tongs and gloves to guard against burns to arms and hands when loading or unloading hot ashing ovens. Stand to one side and cautiously open the oven door halfway. Do not open hot oven before sample has completely ashed as flaming will occur.

11. References

AOAC 942.05. 2000. *Ash of animal feed*. Gaithersburg, MD, USA.

ISO 5984. 2002. *Animal feeding stuffs – Determination of crude ash*. Geneva, Switzerland.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex III, M, *Official Journal of the European Union* L54/1 from 26/02/2009.

ASH INSOLUBLE IN HYDROCHLORIC ACID

1. Principle

Ash insoluble in hydrochloric acid (AIA) is determined gravimetrically as the residue remaining after boiling the ash fraction with hydrochloric acid.

2. Scope

This procedure is applicable for determination of ash in feed ingredients and feeds. This method cannot be used for mineral mixtures.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Incineration dish.
4.2 Analytical electronic balance, accurate to 0.1 mg.
4.3 Muffle furnace, capable of maintaining temperatures to 550 ± 20 °C.
4.4 Desiccator.
4.5 Hot plate.
4.6 Ash-free filter paper.
4.7 Forced-air drying oven capable of maintaining temperature to 103 ± 2 °C. Oven should be equipped with a shelf to allow circulation of air. It should be operated with open vents.

5. Reagents

5.1 3 M hydrochloric acid.

6. Procedure

6.1 Dry incineration dish (4.1) at 103 ± 2 °C for at least 2 hours, remove from oven and cool in a desiccator.
6.2 Weigh the empty incineration dish (4.1) to the nearest 0.1 mg (W1).
6.3 Add approximately 5 g of sample to the dish and weigh to the nearest 0.1 mg (W2).
6.4 Place the dish in preheated muffle furnace (4.3) adjusted to 550 ± 20 °C for 3 hours.
6.5 Inspect visually if the residue is free from carbonaceous particles (see remark 9.1).
6.6 Transfer dish into a desiccator (4.4) and allow to cool down to room temperature (approximately 45 minutes).
6.7 Transfer the ash quantitatively to a beaker using 75 ml 3 M hydrochloric acid (5.1).
6.8 Heat the mixture on a hot plate (4.5) carefully to boiling, and boil for 15 minutes.
6.9 Filter the mixture through ash-free filter paper (4.6) and wash with hot distilled water until the washings are free from acid.

6.10 Transfer the filter paper with the residue to an incineration dish.
6.11 Dry the incineration dish overnight in drying oven (4.7) adjusted to 103 ± 2 °C.
6.12 Place the dish in preheated muffle furnace (4.3) adjusted to 550 ± 20 °C for 2 hours.
6.13 Transfer dish into a desiccator (4.4) and allow to cool to room temperature (approximately 45 minutes).
6.14 Weigh the dish and the residue to the nearest 0.1 mg (W3)

7. Calculation

Percent AIA (% AIA)

$$\% \text{ AIA} = (W3 - W1) \times 100 / (W2 - W1)$$

where,

W1 = weight of empty dish (g),
W2 = weight of the dish and sample (g), and
W3 = weight of dish and residue after incineration (g).

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall not exceed 10% relative to higher value and with a maximum difference of 0.5% units.

9. Remarks

9.1 If the residue contains carbonaceous particles, moisten with distilled water, evaporate the water carefully to dryness at 103 ± 2 °C and incinerate for 1 hour at 550 ± 20 °C.

10. Interferences, troubleshooting and safety

10.1 Do not allow ashed sample to cool below 200 °C in the ashing oven before transferring to a desiccator because sample might absorb moisture.
10.2 Do not allow sample to remain in the desiccator at room temperature for more than 2 hours before weighing.
10.3 Take care when opening the desiccator to avoid loss of the ash.
10.4 Desiccant must be kept fresh and desiccator lid properly sealed with high vacuum grease, a silicone lubricant.
10.5 Ensure that all surfaces with which the crucibles come in contact are clean, especially desiccators where vacuum grease is used.
10.6 Re-tare balance between each weighing.
10.7 The furnace should not be preheated. This prevents rapid ignition of sample and allows slow loss of moisture. Rapid ignition may cause flaming and rapid loss of moisture may cause splattering of sample; both of these situations could result in loss of sample and therefore low results.

10.8 Be aware that some samples foam out of the crucible. If this occurs, repeat the analysis using acid-washed sand and/or use a larger crucible.

HAZARDS: Use long handled tongs and gloves to guard against burns to arms and hands when loading or unloading hot ashing ovens. Stand to one side and cautiously open the oven door halfway. Do not open hot oven before sample has completely ashed as flaming will occur.

11. References

AOAC 942.05. 2000. *Ash of animal feed*. Gaithersburg, MD, USA.

ISO 5985. 2002. *Animal feeding stuffs – Determination of ash insoluble in hydrochloric acid.* Geneva, Switzerland.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex III, N, *Official Journal of the European Union* L54/1 from 26/02/2009.

NITROGEN AND CALCULATION OF CRUDE PROTEIN – KJELDAHL

1. Principle

For determination of nitrogen the sample is digested using sulphuric acid in the presence of a catalyst to convert sample nitrogen to ammonium sulphate. The acid solution is made alkaline with sodium hydroxide solution. The ammonia is distilled and collected in an excess of boric acid solution, followed by titration with sulphuric acid solution. For determination of crude protein nitrogen is multiplied by a factor, 6.25 (or an appropriate factor, see remark 9.3).

2. Scope

The method described is applicable for determination of nitrogen in feeds.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.
4.2 Digestion tubes fitted for the Kjeldahl digestion unit.
4.3 Kjeldahl digestion unit with fume removal manifold.
4.4 Kjeldahl distillation apparatus.
4.5 Titration unit (in sophisticated equipment 4.4 is combined with 4.5).

5. Reagents

5.1 Sulphuric acid, concentrated, 95–98% (w/v), reagent grade.
5.2 Kjeldahl catalyst tablets (see remark 9.5).

5.3 Boric acid, 10 g/litre.
5.4 NaOH solution, 40% (w/v).
5.5 Indicator solution: Methyl red indicator, dissolve 1 g methyl red (sodium salt) in 100 ml methanol or ethanol.
5.6 Hydrochloric acid standard volumetric solution, c = 0.1 M (accurate to 0.1000 M).

6. Procedure

6.1 Digestion

6.1.1 Weigh approximately 1 g sample recording to the nearest 0.1 mg (W) and transfer to the digestion tube (4.2). In each batch use a tube without sample as blank test.

6.1.2 Add two Kjeldahl tablets (5.2) and 20 ml sulphuric acid (5.1). If fuming is a problem, add a few drops of anti-foaming agent.

6.1.3 Place the tubes in a digestion unit (4.3) and connect to the fume removal manifold.

6.1.4 Digest the sample at least 1 hour at 420 ± 20 °C.

6.1.5 Turn the digestion off, remove the tubes and allow to cool for 10–20 minutes.

6.1.6 Add distilled water to each tube to a total volume of approximately 80 ml.

6.2 Distillation and titration

The following procedure describes the manual method of distillation and titration. If an automatic distillation and titration unit is used, follow instructions of the manufacturer.

6.2.1 Place a conical flask containing 25–30 ml of the concentrated boric acid (5.3) under the outlet of the condenser of the distillation unit (4.4) in such a way that the delivery tube is below the surface of the boric acid solution.

6.2.2 Add 50 ml NaOH (5.4) and distill the ammonium by following the instructions of the manufacturer.

6.2.3 Titrate the content of the conical flask with hydrochloric acid standard solution (5.6) (see remark 9.4) after adding a few droplets of indicator solution (5.5) using a titration unit (4.5) and read the amount of titrant used. The endpoint is reached at the first trace of pink colour in the contents.

6.2.4 Record the amount of acid used to the nearest 0.05 ml for the blank test (Vb) and for each sample (Vs).

7. Calculation

Percent Nitrogen (% N)

$$\% N = (Vs - Vb) \times M(HCl) \times 1 \times 14.007 / (W \times 10)$$

where,

Vs	=	ml HCl needed to titrate sample,
Vb	=	ml HCl needed for the blank test,
M(HCl)	=	molarity of HCl,
1	=	the acid factor,
14.007	=	molecular weight of N,
10	=	conversion from mg/g to %, and
W	=	weight of the sample (g).

Calculation percent Crude Protein (% CP):

% CP = % N x F

where,

F = 6.25 for all forages, feeds and mixed feeds,
F = 5.70 for wheat grains, and
F = 6.38 for milk and milk products.

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate.

Use a chemical standard to test the whole procedure, highly purified (> 99.7%) and dried lysine hydrochloride, acetanilide or tryptophan can be used. Ammonium salts can be used to test the distillation and titration step.

Repeatability

The difference between the values of two parallel determinations carried out on the same sample must not exceed:

0.2% in absolute value, for crude protein contents of less than 20%,
1.0% relative to the higher value, for crude protein contents from 20% to 40%, and
0.4% in absolute value, for crude protein contents of more than 40%.

Recovery

The recovery test should be periodically performed and evaluated. The recovery for the partial (distillation only) and the whole procedure (digestion and distillation) should be > 99%.

9. Remarks

9.1 For fresh samples, a higher amount of sample should be used.

9.2 This method can also be used for determination of ammonia in silage samples. The procedure should then be performed without digestion.

9.3 The international conversion factor (F) is based on the average composition of amino acids in proteins in feedstuffs. The true factor can therefore vary between the individual feedstuffs.

9.4 If sulphuric acid is used for the titration, the acid factor used should be 2.

9.5 Catalyst can also be made by the laboratory. It should contain 3.5 g of potassium sulphate and 0.4 g of copper (II) sulphate pentahydrate.

10. Interferences, troubleshooting and safety

10.1 Reagent proportions, heat input and digestion time are critical factors – do not change.

10.2 Dispensers are available for convenient delivery of powdered catalyst mixtures.

SAFETY PRECAUTIONS:

10.3 Handle acid safely. Use acid resistant fume hood. Always add acid to water unless otherwise directed in method. Wear face shield and heavy gloves to protect against acid or alkali splashes. If acid is spilt on skin, immediately wash with large amounts of water.

10.4 Sulphuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely. Use effective fume removal device to protect against acid fumes or alkali dusts or vapours. Always add concentrated sulphuric acid or sodium hydroxide pellets to water, not vice versa. Concentrated sodium hydroxide can quickly and easily cause blindness. If splashed on skin or in eyes, flush with copious amounts of water and seek medical attention.

10.5 Keep baking soda and vinegar handy in case of chemical spills.

10.6 The sulphur oxide fumes produced during digestion are hazardous to breath. Do not inhale.

10.7 Digests must be cooled before the dilution water is added to avoid a violent reaction during which the acid can shoot out of the flask. Likewise, the diluted digest must be cooled before sodium hydroxide is added to avoid a similarly violent reaction.

10.8 Check with local authorities for proper disposal procedures of copper containing waste solution.

11. References

AOAC 984.13. 2000. *Protein (crude) in animal feed and pet food, copper catalyst Kjeldahl method.* Gaithersburg, MD, USA.

AOAC 988.05. 2000. *Protein (crude) in animal feed and pet food: $CuSO_4/TiO_2$ mixed catalyst Kjeldahl method*. Gaithersburg, MD, USA.

AOCS Ba4d-90. *Nitrogen-ammonia-protein modified Kjeldahl method, titanium dioxide + copper sulphate catalyst*. Gaithersburg, MD, USA.

ISO 5983-2. 2009. *Animal feeding stuffs – Determination of nitrogen content and calculation of crude protein content – Part 2: block digestion/steam distillation method.* Geneve, Switzerland.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. *Laying down the methods of sampling and analysis for the official control of feed.* Annex III, C, Official Journal of the European Union L54/1 from 26/02/2009.

NITROGEN AND CALCULATION OF CRUDE PROTEIN – COMBUSTION

1. Principle

Nitrogen is determined by total combustion of the sample at 950 ˚C in the presence of oxygen where the nitrogen is converted to NOx gas (Dumas principle). The NOx is reduced to N_2 which is measured in a thermal conductivity cell. Percent protein is calculated by multiplying the reported nitrogen by 6.25, or the protein conversion factor applicable to the sample type (see remark 9.3).

2. Scope

The method described is applicable for determination of nitrogen in all feeds.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.

4.2 Dumas apparatus, capable of performing the complete determination.

5. Reagents

Chemicals and disposables needed by the equipment should be specified by the manufacture.

5.1 EDTA, Ethylenediaminetetraacetic acid, $C_{10}H_{16}N_2O_8$, powder. This chemical is used as the calibration standard for this analysis.

6. Procedure

6.1 Weigh into the tin cup approximately 0.22 g sample or EDTA (5.1) to the nearest 0.1 mg (W). Liquid feed samples should be weighed into tin capsule by using a pipette (see remark 9.1). Suggested weight for liquids: 0.2–0.5 g depending on expected concentration.

6.2 Carefully close the tin cup and place it in the auto sampler of the equipment (4.2).

6.3 Analyse the standard and samples according to the manufacturer's instructions.

6.4 Calculate the results by using the calibration standard (mostly done automatically by the equipment).

7. Calculation

Percent Nitrogen (% N) is automatically calculated by the equipment (see remark 9.2).

Calculation of crude protein (% CP):

$$\% \text{ CP} = \% \text{ N} \times F$$

where,

F = 6.25 for all forages, feeds and mixed feeds,

F = 5.70 for wheat grains, and

F = 6.38 for milk and milk products.

8. Quality Control

Every batch should include a blank (empty cup or capsule), one sample of high purity lysine hydrochloride, and one or more quality control (QC) samples. A laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm

pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range. Samples should be analysed in duplicate.

Repeatability

The difference between the values of two parallel determinations carried out on the same sample must not exceed:

0.2% in absolute value, for crude protein contents of less than 20%,
1.0% relative to the higher value, for crude protein contents from 20% to 40%, and
0.4% in absolute value, for crude protein contents of more than 40%.

9. Remarks

9.1 For feeds with a very low amount of N, the sample weight should be increased.

9.2 Calculation of N content is based on comparison of the areas of the peaks found for the calibration standard (EDTA) and the samples.

9.3 The international conversion factor is based on the average composition of amino acids in proteins in feedstuffs. The true factor can therefore vary between the individual feedstuffs.

9.4 Nitrogen and therefore the calculated crude protein values can differ slightly from those analysed using the Kjeldahl method.

10. Interferences, troubleshooting and safety

10.1 Systemic errors

10.1.1 Incorrect calibration of blanks and/or calibration standard.

10.1.2 Always recalibrate the instrument following routine maintenance.

10.1.3 Exceeding the counter limits for crucibles/aliquot reagent tubes/reduction tubes by more than 20% may cause a blockage.

10.1.4 Do not overfill the crucible.

10.1.5 If the ballast filter count is around 800 and the calibration and control samples are running consistently low, changing the ballast filter may be necessary.

10.1.6 For specific errors check the manufacturer's instructions.

10.2 Random Errors

10.2.1. Significant deviations from the weight ranges given for a sample. Too little or too much sample may give inaccurate results.

10.2.2 Loss of sample after weighing, due to improper folding of the tin foil cup/capsule or holes/tears in the foil, causes a loss of accuracy in reported results.

10.2.3. Loss of part of the foil cup/tin capsule after taring. Twisting off the edges of the foil cup or the capsule results in weighing errors.

11. References

AOAC 990.03. 2000. *Protein (crude) in animal feed, combustion method.* Gaithersburg, MD, USA.

AOCS Ba4e-93. *Generic combustion method for determination of crude protein. Instruction manual of manufacturer*. Gaithersburg, MD, USA.

CRUDE FAT – ETHER EXTRACT

1. Principle

Fat is extracted from the sample using petroleum ether. The solvent is distilled and the residue is dried and weighed. The fat can be measured with or without previous hydrolysis with hydrochloric acid. The use of previous hydrolysis leads mostly to higher results, especially for heat treated feeds and feeds of animal origin.

2. Scope

The determination of fat can be used for feeds and feed ingredients with a fat content lower than 20%. For feeds with a higher fat content for example oil seeds see remarks 9.1 and 9.2.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.
4.2 Heating apparatus with temperature control.
4.3 Extraction thimbles, free from fat and ether washed.
4.4 Heating apparatus.
4.5 Reflux unit.
4.6 Soxhlet-type extractor.
4.7 Electrically heated vacuum oven.
4.8 Desiccator.
4.9 Buchner funnel connected to suction.

5. Reagents

5.1 Light petroleum ether (boiling point 40–60 °C), purified for fat extraction (i.e. evaporation residue shall be less than 20 mg/litre).
5.2 3 M Hydrochloric acid.

6. Procedure

For determination of fat without hydrolysis, proceed from point 6.2.
For determination of fat with hydrolysis, proceed from 6.1.
This procedure describes a manual method for both determinations (see remark 9.3).

6.1 Hydrolysis
6.1.1 Weigh at least 5 g of the sample into a beaker or conical flask and record the weight to nearest 0.1 mg (W1).
6.1.2 Add 100 ml hydrochloric acid (5.2) and silicon carbide chips to the sample and

cover the beaker with a watch glass or fit the conical flask with a reflux condenser (4.5).

6.1.3 Bring the mixture to a gentle boil over a heating apparatus (4.4) and maintain it for 1 hour. Swirl every 10 minutes to prevent the product sticking to the sides of the container.

6.1.4 Filter the mixture through a fat-free double filter paper in a Buchner funnel (4.9) with suction.

6.1.5 Wash the residue with cold distilled water until a neutral filtrate is obtained.

6.1.6 Transfer the filter paper with the residue carefully into an extraction thimble (4.3) and dry in a vacuum oven (4.7) for 60 minutes at 80 ± 2 °C.

6.1.7 Remove the thimble from the oven and cover with a fat-free wad cotton of wool. Follow the procedure given in 6.2.2

6.2 Extraction

6.2.1 Weigh at least 5 g of the sample to the nearest 0.1 mg (W1) into the extraction thimble (4.3) and cover with a fat-free wad of cotton wool. This step should only be performed for the determination of fat without hydrolysis

6.2.2 Transfer some silicon carbide chips to a dry flask and weigh to the nearest 0.1 mg (W2), add 95 ml petroleum ether (5.1).

6.2.3 Place the thimble in the extractor (4.6) and connect it to the dry flask (6.2.2) and reflux unit (4.5).

6.2.4 Extract for 6 hours with petroleum ether and regulate the heating apparatus (4.4) to obtain at least 10 siphonings per hour. Or follow the manufacturer's guidelines.

6.2.5 Distill the solvent until the flask is nearly free from the solvent, leave overnight in a fume hood to ensure all solvent is evaporated.

6.2.6 Dry the flask with residue for 1.5 hour in a vacuum oven (4.7) at 80 ± 2 °C .

6.2.7 Cool in a desiccator (4.8) and weigh to the nearest 0.1 mg (W3).

7. Calculation

Percent Crude Fat with or without hydrolysis:

$$\% \text{ Crude Fat} = (W3 - W2) \times 100 / W1$$

where,

W1 = initial sample weight in grams,

W2 = tare weight of flask in grams, and

W3 = weight of flask and fat residue in grams.

Reported results should note if hydrolysis was used.

8. Quality Control

Include a reagent blank and one or more quality control (QC) samples in each run, choose QC samples by matching analyte levels and matrices of QC samples to the samples in the batch. Include at least one set of duplicates in each run. For example a bulk sample of oil seed cake can be run as a quality control sample with each batch, average 15–20 replicates and allow ± 2 SD as an acceptable range. Samples should be analysed in duplicate.

NOTE: Quality control sample for fat determination should be changed every 6 months due to instability of fats. The control sample should be kept at 2–8 °C.

The difference between sample duplicates should be lower than 0.25% in absolute terms in the procedure without hydrolysis and 0.50% in the procedure with hydrolysis.

9. Remarks

9.1 Samples with a high fat content should undergo a preliminary extraction with light petroleum ether by the procedure described in the SOP sample preparation (see page 42–47).

9.2 For oil seeds, a double extraction with petroleum ether is recommended. An alternative method for these products is specified in ISO 659:2009.

9.3 Semi-automatic equipment for the fat determination is available. In this case follow the manufacturer's instructions.

10. Interferences, troubleshooting and safety

10.1 Ether has an extremely low flash point.

10.2 Avoid inhaling ether vapours.

10.3 Store ether in metal containers.

10.4 Handle open containers (reagent containers and fat beakers) in a fume hood.

10.5 Conduct the extractions in a well-ventilated area.

10.6 Peroxides can accumulate in open containers of ether. These are explosive and shock sensitive. Check each container opened for more than 30 days for peroxides. Ether-containing peroxides must be disposed of with special techniques. Dipsticks are available to check peroxide levels.

10.7 Electrical equipment shall be grounded. Extractors should be spark-proof.

10.8 Make sure all ether is evaporated from the beakers before placing them in the oven to avoid a fire or explosion, leave beakers in a fume hood overnight.

11. References

AOAC 920.39. 2000. *Fat (crude) or ether extract in animal feed.* Gaithersburg, MD, USA.

ISO 6492. 1999. *Animal feeding stuffs – Determination of fat content*. Geneva, Switzerland.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. *Laying down the methods of sampling and analysis for the official control of feed.* Annex III, H, *Official Journal of the European Union* L54/1 from 26/02/2009.

CRUDE FIBRE – FILTRATION METHOD

1. Principle

The sample after defatting is sequentially treated with boiling dilute sulphuric acid, and with boiling potassium hydroxide solution. The loss in mass resulting from incineration corresponds to the mass of crude fibre.

2. Scope

The method described is applicable for determination of feeds with a crude fibre content higher than 1%. If sample contains >10% fat, extract fat with petroleum ether prior to beginning analysis.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.
4.2 Glass filter crucibles, P100.
4.3 Heating apparatus.
4.4 Filtration equipment, connected to a vacuum system, for example a Fibertec system.
4.5 Desiccator.
4.6 Drying oven ventilated, capable of being maintained at a temperature of 103 ± 2 °C.
4.7 Muffle furnace, capable of being maintained at a temperature of 550 ± 20 °C.

5. Reagents

5.1 Petroleum ether (boiling point 40 to 60 ˚C).
5.2 Sulphuric acid, 0.15 M.
5.3 Acetone technical quality.
5.4 Potassium hydroxide, 0.23 M.

6. Procedure

6.1 Pretreatment
- 6.1.1 To each P100-crucible (4.2) weigh 1 g of the sample to the nearest 0.1 mg (W1).
- 6.1.2 Place the crucibles in the filtration equipment (4.4) and add approximately 30 ml of petroleum ether (5.1) to each crucible and filter using vacuum.
- 6.1.3 Repeat the washing two times.
- 6.1.4 Dry the residue in air and transfer quantitatively to a beaker.

6.2 Digestion
- 6.2.1 Add to each beaker 150 ml sulphuric acid (5.3) and boil for 30 ± 1 minutes. If foaming occurs, add a few drops of anti-foaming agent.
- 6.2.2 Filter the mixture through a crucible (4.2) using vacuum (4.4).
- 6.2.3 Wash the residue 5 times, each time with 10 ml of hot distilled water.
- 6.2.4 Add a volume of acetone (5.4) to just cover the residue. Remove the acetone after a few minutes by applying slight suction.
- 6.2.5 Transfer the residue quantitatively to a beaker.
- 6.2.6 Add to each beaker 150 ml potassium hydroxide (5.1.8) and boil for 30 ± 1 minute.
- 6.2.7 Filter the mixture through a crucible (4.2) using vacuum (4.4).
- 6.2.8 Wash the residue with hot distilled water until the rinsings are neutral.

6.2.9 Wash the residue 3 times under vacuum, each time with 30 ml of acetone (5.4). Dry the residue by suction after each washing.

6.3 Drying and incineration

6.3.1 Put the crucibles in an oven (4.6) adjusted to 103 ± 2 °C and dry for 4.0 hours. The drying time starts when the oven has reached 103 °C.

6.3.2 Place the crucibles in a desiccator and allow to cool.

6.3.3 Weigh the crucible directly after removing from the desiccator (4.5) to the nearest 0.1 mg (W2).

6.3.4 Place the crucibles in a muffle furnace (4.7), and incinerate the samples for 2 hours at 550 ± 20 °C. The incineration time starts when the furnace has reached 550 °C.

6.3.5 Place the crucibles in a desiccator (4.5) and allow to cool.

6.3.6 Weigh the crucible directly after removing from the desiccator to the nearest 0.1 mg (W3).

7. Calculation

Percent Crude fibre (% CF):

$$\% CF = (W2 - W3) \times 100 / W1$$

where,

W1 = weight of the sample (g),
W2 = weight crucible and residue after drying (g), and
W3 = weight crucible and residue after incineration (g).

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range. Samples should be analysed in duplicate.

The difference between the values of two parallel determinations carried out on the same sample shall not exceed:

0.3% in absolute terms for contents lower than 10%, and
3% relative to higher value for contents equal to or higher than 10%.

9. Remarks

9.1 For the determination of crude fibre there are other methods available that apply automatic washing of bags with the described reagents. To perform these methods follow the instructions of the manufacturer. The use of these methods can lead to different results compared to those observed with the filtration method.

9.2 If filtration problems are encountered a layer of sea sand can be used as a filtration aid.

10. References

ISO 6865. 2000. *Animal feeding stuffs – Determination of crude fibre content – Method with intermediate filtration*. Geneva, Switzerland.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. *Laying down the methods of sampling and analysis for the official control of feed.* Annex III, I, *Official Journal of the European Union* L54/1 from 26/02/2009.

NEUTRAL DETERGENT FIBRE (NDF) – FILTRATION METHOD

1. Principle

The sample after defatting is boiled with the neutral detergent reagent (NDF) followed by treatment with α-amylase in a buffer solution to dissolve the remaining starch. The loss in weight resulting from incineration of the dried residue corresponds to the weight of NDF.

2. Scope

The method described is applicable for determination of feeds with an NDF content higher than 1%.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.
4.2 Glass filter crucibles, P100 or equivalent.
4.3 Heating apparatus.
4.4 Filtration equipment, connected to a vacuum system, for example a Fibertec system.
4.5 Desiccator.
4.6 Drying oven ventilated, capable of being maintained at a temperature of 103 ± 2 °C.
4.7 Muffle furnace, capable of being maintained at a temperature of 550 ± 20 °C.

5. Reagents

5.1 Acetone technical quality.
5.2 Neutral detergent solution (NDS), which contains in 1 litre distilled water:
30 g Sodium-lauryl sulphate (=dodecyl-Na-sulphate)
6.81 g Sodium tetra borate $Na_2B_4O_7.10\ H_2O$.
4.56 g di-Sodium hydrogen phosphate Na_2HPO_4 or 5.72 g $Na_2HPO_4.H_2O$
18.61 g Ethylene diamine tetra acetic acid, di-sodium salt.
10 ml 2-ethoxy-ethanol
Adjust the pH of the solution to 6.9–7.1 using sodium hydroxide or hydrochloric acid.
5.3 Amylase solution. Dissolve 2.5 mg heat stable amylase in 60.6 ml of 0.1 M disodium hydrogen phosphate (Na_2HPO_4) and 39.2 ml of 0.1 M potassium dihydrogen phosphate (KH_2PO_4).

6. Procedure

6.1 Pretreatment

6.1.1 Weigh each P100-crucible (4.2).

6.1.2 Weigh 0.5 g of the sample to the nearest 0.1 mg (W1) in a crucible.

6.1.3 Place the crucibles in the filtration equipment (4.4) and add approximately 30 ml of acetone (5.1) to each crucible and filter using vacuum.

6.1.4 Repeat the washing two times.

6.1.5 Dry the residue in air and transfer quantitatively to a beaker.

6.2 Digestion

6.2.1 Add to each crucible 50 ml NDS (5.2) and boil for 60 ± 1 minute under reflux, if foaming occurs, add a few drops of anti-foaming agent.

6.2.2 Filter the mixture through a crucible (4.2) using vacuum (4.4).

6.2.3 Wash the residue two times, each time with 10 ml of hot distilled water.

6.2.4 Add a volume of acetone (5.1) to just cover the residue. Remove the acetone after a few minutes by applying slight suction.

6.2.5 Transfer the residue quantitatively to a beaker.

6.2.6 Add to each beaker 1–2 ml of α-amylase solution (5.3) and then 30 ml of boiling distilled water. Allow to stand 5–10 minutes.

6.2.7 Transfer the mixture to a crucible (4.2) and wash twice with hot distilled water and twice with acetone (5.1) until the washing is clean. Dry the residue by suction after each washing.

6.3 Drying and incineration

6.3.1 Place the crucibles in an oven (4.6) adjusted to 103 ± 2 °C and dry them for at least 4 hours. The drying time starts when the oven has reached 103 °C.

6.3.2 Place the crucibles in a desiccator (4.5) and allow to cool.

6.3.3 Weigh the crucible directly after removing from the desiccator to the nearest 0.1 mg (W2).

6.3.4 Place the crucibles in the muffle furnace (4.7), and incinerate for 2 hours at 550 °C ± 20 °C. The incineration time starts when the furnace has reached 550 °C.

6.3.5 Place the crucibles after incineration in a desiccator (4.5) and allow to cool.

6.3.6 Weigh the crucible directly after removing from the desiccator to the nearest 0.1 mg (W3).

7. Calculation

Percent NDF (%NDF):

$$\%NDF = (W2 - W3) \times 100 / W1$$

where,

W1 = weight of the sample (g),

W2 = weight crucible and residue after drying (g), and

W3 = weight crucible and residue after incineration (g).

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples

to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall not exceed: 0.3% in absolute terms for contents lower than 10% and 3% relative to higher value for contents equal to or higher than 10%.

9. Remarks

9.1 For determination of NDF, methods are also available that apply automatic washing of bags with the described reagents. To perform these methods follow the instructions of the manufacturer. The use of these methods can lead to different results compared to those found with the filtration method.

9.2 An additional incubation with protease can be used to remove remains of non-soluble proteins in the residue.

9.3 Because of the treatment with α-amylase in a buffer solution to dissolve the remaining starch the fibre fraction is also termed 'aNDF'. The use of α-amylase can be omitted if the samples, (for example straws and hays) are not rich in starch.

9.4 If filtration problems are encountered a layer of sea sand can be used as a filtration aid.

10. References

Robertson, J.B. & Van Soest, P.J. 1981.The detergent system of analysis and its application to human foods, In *The Analysis of Dietary Fibre in Food*. Vol 3. Chapter 8, (W.P.T. James and O. Theander, eds.), Marcel Dekker, Inc.: New York.

Van Soest, P.J. & Robertson, J.B. 1985. *Analysis of forage and fibrous foods*. A Laboratory Manual for Animal Science 613. Cornell University, Ithaca, New York, USA.

ACID DETERGENT FIBRE (ADF) AND LIGNIN (ADL) – FILTRATION METHOD

1. Principle

The sample after defatting is boiled with an acid detergent solution. The loss in weight resulting from incineration of the dried residue corresponds to the weight of ADF.

For the determination of ADL, additional boiling with concentrated sulphuric acid should be performed before measuring the loss in weight resulting from incineration of the dried residue.

2. Scope

The method described is applicable for determination of feeds with an ADF or ADL content higher than 1%.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.
4.2 Glass filter crucibles, P100 or equivalent.
4.3 Heating apparatus.
4.4 Filtration equipment, connected to a vacuum system, for example a Fibertec system.
4.5 Desiccator.
4.6 Drying oven ventilated capable of being maintained at a temperature of 103 ± 2 °C.
4.7 Muffle furnace capable of being maintained at a temperature of 550 ± 20 °C.

5. Reagents

5.1 Acetone technical quality.
5.2 Acid detergent solution (ADS), which contains 20 g cetyl trimethylammonium bromide in 1 litre of 0.5 M sulphuric acid.
5.3 72% Sulphuric acid (w/w).

6. Procedure

6.1 Pretreatment
- 6.1.1 Weigh empty crucible (4.2) to the nearest 0.1 mg (see remark 9.4).
- 6.1.2 Weigh 1 g of the sample to the nearest 0.1 mg (W1) into a crucible (4.2).
- 6.1.3 Place the crucibles in filtration equipment (4.4) and add approximately 30 ml of acetone (5.1) to each crucible and filter using vacuum.
- 6.1.4 Repeat the washing two times.
- 6.1.5 Dry the residue in air and transfer quantitatively to a beaker.

6.2 Digestion (ADS-step)
- 6.2.1 Add to each beaker 50 ml ADS (5.2) and boil for 60 ± 1 minutes whilst under refluxing. If foaming occurs, add a few drops of anti-foaming agent.
- 6.2.2 Filter the mixture through a crucible (4.2) using vacuum.
- 6.2.3 Wash the residue two times, each with 10 ml of hot distilled water.
- 6.2.4 Add a small amount of acetone (5.1) to just cover the residue. Remove the acetone after a few minutes by applying slight suction.

6.3 First drying
- 6.3.1 Place the crucibles in an oven (4.6) adjusted to 103 ± 2 °C and dry for at least 4 hours. The drying time starts when the oven has reached 103 °C.
- 6.3.2 Place the crucibles in a desiccator (4.5) and allow to cool.
- 6.3.3 Weigh the crucible directly after removing from the desiccator to the nearest 0.1 mg (W2).

6.4 Digestion (ADL-step).

NOTE: **Wear Safety Goggles and Gloves When Working With 72% sulphuric acid.**
- 6.4.1 Add 10 ml of 72% sulphuric acid (5.3) to each crucible and stir carefully with a glass rod in order to break up all clumps (see remark 9.3)
- 6.4.2 Fill crucibles half full with the acid and stir every 30 minutes.
- 6.4.3 After 3 hours filter off as much of the acid as possible with vacuum and wash the contents with hot distilled water until free of acid.

6.5 Second drying

6.5.1 Place the crucibles in an oven (4.6) adjusted to 103 ± 2 °C and dry for at least 4 hours. The drying time starts when the oven has reached 103 °C.

6.5.2 Place the crucibles in a desiccator (4.5) and allow to cool.

6.5.3 Weigh the crucible directly after removing from the desiccator to the nearest 0.1 mg (W3).

6.6 Incineration

6.6.1 Place the crucibles in the muffle furnace (4.7), and incinerate the samples for 2 hours at 550 °C ± 20 °C. The incineration time starts when the furnace has reached 550 °C.

6.6.2 Place the crucibles in a desiccator (4.5) and allow to cool.

6.6.3 Weigh the crucible directly after removing from the desiccator to the nearest 0.1 mg (W4).

7. Calculation

Percent ADF (% ADF):

$$\% \text{ ADF} = (W2 - W4) \times 100 / W1$$

Percent ADL (% ADL)

$$\% \text{ ADL} = (W3 - W4) \times 100 / W1$$

where,

W1 = weight of sample (g),
W2 = weight of crucible and residue after first drying (g),
W3 = weight of crucible and residue after second drying (g), and
W4 = weight of crucible and residue after incineration (g).

8.Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range. Samples should be analysed in duplicate.

The difference between the values of two parallel determinations carried out on the same sample shall not exceed:

0.3% in absolute terms for contents lower than 10%.

3% relative to the higher value for contents equal to or higher than 10%.

9. Remarks

9.1 For the determination of ADF, methods are also available that apply automatic washing of bags with the described reagents. To perform these methods follow the instructions of the manufacturer. The use of these methods can lead to different results compare to those found with the filtration method.

9.2 An additional incubation with amylase can be used to remove remains of non- soluble starch in the residue.

9.3 Take care not to damage the glass frit in crucible when using glass rod.

9.4 Some laboratories omit the incineration step (6.6). In such a case, acid detergent residue (ADR) is determined which is the sum of ADF and the remaining ash traction.

Percent ADR (% ADR):

$$\% \text{ ADR} = (W2 - W0) \times 100 / W1$$

where,

W0 is the weight of the empty crucible obtained in step 6.1.1. If the ash correction is not performed, this should be stated in the report.

9.5 If filtration problems are encountered a layer of sea sand can be used as a filtration aid.

10. References

AOAC 973.18. 2010. *Fiber (acid detergent) and lignin (H_2SO_4) in animal feed*. Gaithersburg, MD, USA.

Robertson, J.B. & Van Soest, P.J. 1981. The detergent system of analysis and its application to human foods, In *The Analysis of Dietary Fibre in Food*, Vol 3. Chapter 8, (W.P.T. James and O. Theander, eds.). Marcel Dekker, Inc.: New York.

Van Soest, P.J. & Robertson, J.B. 1985. Analysis of forage and fibrous foods. In *A Laboratory Manual for Animal Science* 613. Cornell University, Ithaca, New York, USA.

STARCH – ENZYMATIC

1. Principle

Starch is determined by previous extraction of the sample in 40% ethanol to remove soluble sugars, followed by solubilisation of starch using dimethyl sulphoxide (DMSO), and then quantitatively converting starch into glucose by amyloglucosidase. The released glucose is spectrometrically measured by the hexokinase method.

2. Scope

The method can be used for all feeds with a starch content above 2% (see remark 9.1).

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Balance, analytical accurate to 0.1 mg.
4.2 Bench centrifuge capable of achieving 3000 *g*.
4.3 Water bath capable of being maintained at 60 ± 1 °C.
4.4 Water bath, boiling 95 to 100 °C.
4.5 Vortex mixer.
4.6 pH meter.
4.7 Spectrophotometer.

5. Reagents

5.1 Ethanol, 40%
Mix ethanol and distilled water in a volume ratio of 2:3.

5.2 Dimethyl sulphoxide (DMSO) 90%
Mix pure DMSO and distilled water in a volume ratio of 9:1.

5.3 Hydrochloric acid, 12 M.

5.4 Aqueous sodium Hydroxide solution, 4 M
Weight 40 g NaOH in 250 ml volumetric flask and make the volume up to the mark with distilled H_2O.

5.5 Acetic acid solution, 2 M
Add to a 500 ml volumetric flask about 200 ml distilled water and 59 ml acetic acid. Dilute to the mark with distilled water.

5.6 Sodium acetate solution, 2 M
Dissolve 82 g sodium acetate in approximately 300 ml distilled water in a 500 ml volumetric flask and then dilute to the mark with distilled water.

5.7 Sodium acetate buffer, 2 M, pH = 4.8
Mix 41 ml acetic acid solution (5.5) with 59 ml of sodium acetate solution (5.6). Check pH and adjust if necessary.

5.8 Potassium hexacyanoferrate (II) solution, 0.25 M
In a 1-L volumetric flask dissolve 106 g of potassium hexacyanoferrate trihydrate in distilled water and then dilute to the mark with distilled water.

5.9 Zinc acetate (1 M) in 0.5 M acetic acid
In a 1-L volumetric flask dissolve 219.5 g zinc acetate dehydrate and 30 g glacial acetic acid in distilled water and dilute to the mark with distilled water.

5.10 Iodine solution in potassium iodide
In a 1-L volumetric flask dissolve 12.7 g iodine and 24 g potassium iodide in distilled water and dilute to the mark with distilled water.

5.11 Standard Glucose Solution
For samples containing 200–1000 g/kg starch. Prepare three separate glucose solutions (0.0194 M). In each 100 ml volumetric flask dissolve 350 mg ± 1 mg anhydrous glucose, dilute to the mark with distilled water.
For sample containing 40–200 g/kg starch. Prepare three separate glucose solutions (0.0039 M). In each 500 ml volumetric flask dissolve 350 mg ± 1 mg anhydrous glucose, dilute to the mark with distilled water.
Prepare fresh glucose solutions daily.

5.12 Amyloglucosidase solution 160 U/ml (AMG)
Dissolve in a mixture of 9 ml distilled H_2O + 1 ml sodium acetate buffer (5.7), 267 mg of AMG (EC 3.2.1.3 (*Aspergillus niger*, Roche Diagnostics, No.1 202 367, 6 U/mg). Follow storage instructions provided by the manufacturer.
NOTE: 1 unit of AMG will release 1 µmol of glucose from glycogen in 1 minute at 25 °C at pH 4.75.

5.13 D-Glucose UV test set for quantifying glucose enzymatically with the hexokinase method (R-Biopharm, No. 10 716 251 035) according to the manufacturer's instructions. Unused kits may be stored for 1 year at 4 °C.

Other commercial test kits for the determination of glucose based on the hexokinase method can also be used.

6. Procedure

6.1 Free Sugar extraction

6.1.1 Weigh approximately 0.2 g of sample to the nearest 0.1 mg (W) in a centrifuge tube (4.2).

6.1.2 Add 10 ml 40% ethanol (5.1), vortex well and shake for 10 minutes.

6.1.3 Centrifuge for 10 minutes at 3000 *g* and discard the supernatant.

6.1.4 Repeat step 6.1.2 and 6.1.3.

6.2 Disintegration of the starch

Include an empty tube as a blank at this stage of the method.

6.2.1 Add 15 glass pearls to the centrifuge tube (6.1.4) and add 10.0 ml DMSO solution (5.2) with continuous vortex mixing (see remark 9.3), close the tube with a screw cap.

6.2.2 Shake the tubes in a boiling water bath for 30 minutes.

6.2.3 Take the tubes out, allow to cool, add 1.7 ml hydrochloric acid (5.3) using a pipette and mix well.

6.2.4 Close the tubes and shake for 30 minutes in a water bath adjusted to 60 °C ± 1 °C.

6.2.5 Cool the tube and quantitatively transfer to 100 ml volumetric flask.

6.2.6 Add 5.0 ml aqueous sodium hydroxide (5.4) and 2.5 ml sodium acetate buffer (5.7) and thoroughly mix the solution. Adjust the pH, if necessary, to 4.8 ± 0.1 with dilute hydrochloric acid or sodium hydroxide. Make the volume up to 100 ml with distilled water.

6.3 Enzymatic conversion of the starch to glucose

6.3.1 Pipette 5.00 ml of the solution (6.2.6) into another centrifuge tube and add 0.125 ml of AMG enzymatic solution (5.12). Close the tube and mix thoroughly.

6.3.2 Incubate for 16 hours in a water bath adjusted to 60 °C ± 1 °C.

6.3.3 Thereafter, place the tubes in a boiling water bath for 15 minutes to stop the reaction.

6.3.4 Cool the tubes to ambient temperature and add 0.125 ml of potassium hexacyanoferrate (II) solution (5.8) and shake for 1 minute.

6.3.5 Add 0.125 ml zinc acetate solution (5.9), shake for 1 minute, centrifuge for 10 minutes at 3000 *g*.

6.3.6 Transfer the supernatant to another tube (see remark 9.4).

6.4 Enzymatic determination of glucose content

6.4.1 Dilute 0.5 ml of the supernatants (6.3.6), standards (5.11), and the blank respectively with 9.5 ml of distilled water, for starch content > 200 g/kg. In the case of a lower starch content, higher volume of the supernatants should be taken and volume made to 10 ml with distilled water.

6.4.2 Measure the glucose in the dilute solutions (6.4.1) by the hexokinase method by following the manufacturer's instructions. Measure the absorbance at 340 nm using a spectrophotometer.

7. Calculation

Calculate the glucose content in the measured solution by linear regression.
Percent of starch is calculated as:

$$\% \text{ Starch} = (C \times V \times DF \times 162/180) / (W \times 10)$$

where,

C = concentration glucose in measured solution (mg/l),
V = volume of solution (in litre, i.e. 0.1),
DF = dilution factor (4 or 10),
162/180 = factor to convert glucose to starch,
W = weight of the sample (g), and
10 = factor to convert g/kg to %

8. Quality control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall be < 0.5% + 1% relative to the mean of the duplicate values. As an example: If mean of the duplicates is 12%, the difference between duplicates should not be higher than 0.5% plus 0.12% (1% of 12%) i.e. 0.62%.

9. Remarks

9.1 Technological treatment can lead to degradation of starch to glucose oligomers (dextrins) that are not measured with this method.

9.2 It is advisable to grind sample to pass through a sieve of pore size 0.5 mm for the starch determination.

9.3 Vigorous homogenisation/mixing during addition of DMSO is necessary to prevent the formation of lumps.

9.4 To check if all starch is converted, an iodine test can be performed. Add a few ml of distilled water to the tube, boil for 10 minutes, cool and add 2 ml of iodine (5.10). A blue colour indicates the presence of starch and therefore incomplete conversion. In this case, the analysis should be repeated.

9.5 If the starch content of the sample is unknown, the measurement should be performed by using two dilution rates. The results should match at least within the demands of duplicates.

10. Interferences, troubleshooting and safety

10.1 Reagents and solutions should be made with laboratory grade water.

10.2 Efficiency of enzymes and conditions must be validated.

10.3 Concentrations of > 0.5% glucose in the enzyme preparations can cause background absorbance readings and interfere with sample measurements.

11. References

ISO 15904. 2004. *Animal feeding stuffs – Enzymatic determination of total starch content*. Geneva, Switzerland.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. *Laying down the methods of sampling and analysis for the official control of feed.* Annex III, L, *Official Journal of the European Union* L54/1 from 26/02/2009 – Polarimetric method for the determination of starch.

REDUCING SUGAR – LUFF SCHOORL METHOD

1. Principle

The sugars are extracted in dilute ethanol and the solution is clarified with Carrez solutions I and II. After removing the ethanol, the reducing sugars are determined using Luff-Schoorl reagent.

This method determines the amount of reducing sugars and total sugars after inversion, expressed as glucose or where appropriate as sucrose, converting by the factor 0.95.

2. Scope

It is applicable to feedstuffs including compound feeds.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Balance, analytical accurate at 0.1 mg.

4.2 Mixer (tumbler): approximately 35 to 40 rpm.

4.3 Titration unit.

5. Reagents

5.1 Ethanol solution 40% (v/v), density: 0.948 g/ml at 20 °C, neutralised using 1% phenolphthalein solution (5.18). (If necessary this can be achieved by placing ethanol solution on a stirring plate and adding 5 M NaOH dropwise until the water turns just pink. Add 0.5 M H_2S0_4 dropwise, carefully, until the pink just clears and then make it just pink again with 0.5 M NaOH).

5.2 Carrez solution I

Dissolve 21.9 g of zinc acetate $Zn(CH_3COO)_2.2\ H_2O$ and 3 g of glacial acetic acid in distilled water. Make up to 100 ml with distilled water.

5.3 Carrez solution II

Dissolve 10.6 g of potassium ferrocyanide $K_4Fe(CN)_6.3\ H_2O$ in distilled water. Make up to 100 ml with distilled water.

5.4 Methyl orange solution, 0.1% (w/v).

5.5 4 M Hydrochloric acid.

5.6 0.1 M Hydrochloric acid.

5.7 0.1 M Sodium hydroxide solution.

5.8 Copper sulphate solution

Dissolve 25 g of copper sulphate, $CuSO_4.5\ H_2O$, free from iron, in 100 ml of distilled water.

5.9 Citric acid solution

Dissolve 50 g of citric acid, $C_6H_8O_7.H_2O$ in 50 ml of distilled water.

5.10 Sodium carbonate solution

Dissolve 143.8 g of anhydrous sodium carbonate in approximately 300 ml of warm distilled water. Leave to cool.

5.11. Luff-Schoorl reagent

While stirring, carefully pour the citric acid solution (5.9) into the sodium carbonate solution (5.10). Add the copper sulphate solution (5.8) and make up to 1 litre with distilled water. Leave to settle overnight and filter. Check concentration of the reagent thus obtained (Cu 0.05 M; Na_2CO_3 1 M), see (6.4) last paragraph. The pH of the solution should be approximately 9.4.

5.12 Sodium thiosulphate solution, 0.1 M

Dissolve 24.8 g of $Na_2S_2O_3.5H_2O$ in a 1-L flask and make up to volume with distilled water.

5.13 Starch solution

Add a mixture of 5 g of soluble starch in 30 ml of distilled water to 1 litre of boiling distilled water. Boil for 3 minutes, leave to cool and if necessary add 10 mg of mercuric iodide as a preservative.

5.14 Sulphuric acid, 3 M.

5.15 Potassium iodide solution 30% (w/v).

5.16. Granulated pumice stone (can be obtained for example from Sigma-Aldrich) boiled in hydrochloric acid, washed with distilled water and dried.

5.17. 3-methylbutan-1-ol.

5.18. 1% Phenolphthalein

Weigh 1 g phenolpthalein into a 200 ml beaker, add 60 ml drum alcohol, 40 ml distilled water and mix.

6. Procedure

6.1 Extraction of sample

6.1.1 Weigh approximately 2.5 g of sample to the nearest 0.1 mg and place in a 250 ml volumetric flask.

6.1.2 Add 200 ml of ethanol (5.1) and shake for 1 hour.

6.1.3 Add 5 ml of Carrez solution I (5.2) and stir for approximately 30 seconds.

6.1.4 Add 5 ml of Carrez solution II (5.3) and stir for 1 minute.

6.1.5 Make up to 250 ml with ethanol (5.1), mix thoroughly and filter.

6.1.6 Remove 200 ml of the filtrate and evaporate to approximately half volume in order to remove most of the ethanol.

6.1.7 Transfer the evaporated residue quantitatively to a 200 ml volumetric flask using warm distilled water, cool, make up to the mark with distilled water, mix thoroughly and filter if necessary.

This solution is used to determine the amount of reducing sugars and after inversion, of total reducing sugars.

6.2 Determination of reducing sugars

6.2.1 Using a pipette, remove not more than 25 ml of the solution (6.1.7) containing less than 60 mg of reducing sugars expressed as glucose (see remark 9.4).

6.2.2 Determine the content of reducing sugar in this solution by the Luff-Schoorl method (6.4).

6.3 Determination of total reducing sugars after inversion

6.3.1 Using a pipette, transfer 50 ml of solution (6.1.7) into a 100 ml volumetric flask.

6.3.2 Add a few drops of methyl orange solution (5.4), carefully and while stirring continuously, add hydrochloric acid (5.5) until the liquid turns a definite red.

6.3.3 Add 15 ml of hydrochloric acid (5.6) and immerse the flask in a bath containing vigorously boiling water for 30 minutes.

6.3.4 Cool rapidly to approximately 20 °C and add 15 ml of sodium hydroxide solution (5.7). An ice bath may be used for the cooling.

6.3.5 Make up to 100 ml with distilled water and mix thoroughly.

6.3.6 Remove not more than 25 ml containing less than 60 mg of reducing sugars expressed as glucose (see remark 9.4).

6.3.7 Determine the reducing sugar content in this solution by the Luff-Schoorl method (6.4).

6.4 Titration by the Luff-Schoorl method

6.4.1 Using a pipette, transfer 25 ml of Luff-Schoorl reagent (5.11) into a 300 ml Erlenmeyer flask.

6.4.2 Add exactly 25 ml of the clarified sugar solution.

6.4.3 Add 2 granules of pumice stone (5.16), heat over a free flame of medium height and bring the liquid to the boil in approximately 2 minutes. While heating on the flame keep the solution stirring by hand.

6.4.4 Place the Erlenmeyer flask immediately on an asbestos-coated wire gauze with a hole of approximately 6 cm in diameter under which a flame has been lit. The flame should be regulated in such a way that only the base of the Erlenmeyer flask is heated.

6.4.5 Fit a reflux condenser to the Erlenmeyer flask. Boil for exactly 10 minutes.

6.4.6 Cool immediately in cold water and after approximately 5 minutes start the titration.

6.4.7 Add 10 ml of potassium iodide solution (5.15) and immediately afterwards (carefully, because of the risk of abundant foaming) add 25 ml of sulphuric acid (5.14).

6.4.8 Titrate with sodium thiosulphate solution (5.12) until a dull yellow colour appears, add the starch indicator (5.13) and complete the titration and record the volume used (Vs).

6.4.9 Carry out the same titration on an accurately measured mixture of 25 ml of Luff-Schoorl reagent (5.8), 25 ml of distilled water, 10 ml of potassium iodide solution (5.15) and 25 ml of sulphuric acid (5.14) without boiling and record the volume used (Vb).

TABLE 2
Values for 25 ml of Luff-Schoorl reagent
ml of $Na_2S_2O_3$ (0.1 M), 2 minutes heating, 10 minutes boiling

$Na_2S_2O_3$	Glucose, fructose, invert sugar
0.1 M	$C_6H_{12}O_6$
ml difference	*mg*
1	2.4
2	4.8
3	7.2
4	9.7
5	12.2
6	14.7
7	17.2
8	19.8
9	22.4
10	25
11	27.6
12	30.3
13	33
14	35.7
15	38.5
16	41.3
17	44.2
18	47.1
19	50

7. Calculation

Calculate for each sample the difference between the titration volumes used for the blank (Vb) and the sample (Vs). Use Table 2 to translate this difference (expressed in the first column) to the corresponding amount of glucose in mg (expressed in the second column). Express the result as a percentage of the sample:

Content glucose [%] = amount glucose [mg] / (weight sample [g] x 10)

Example: For each determination the two volumes taken correspond to a sample of 250 mg. In the first case 17 ml of sodium thiosulphate solution (0.1 M) corresponding to 44.2 mg of glucose consumed; and in the second 11 ml corresponding to 27.6 mg of glucose.

The difference is 16.6 mg of glucose. The content of reducing sugars (excluding lactose), calculated as glucose, is therefore:

Content glucose [%] = 16.6 mg / (0.25 g x 10 g) = 6.64%

NOTE: The difference between the content of total reducing sugars after inversion (expressed as glucose) and the content of reducing sugars (expressed as glucose) when, multiplied by 0.95 gives the percentage content of sucrose.

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of the two parallel determinations carried out on the same sample should be < 12% relative to the higher value.

9. Remarks

9.1 In the case of a feed that is rich in molasses, and other feeds that are not particularly homogeneous, weigh 20 g and place with 500 ml of distilled water in a 1-L volumetric flask. Mix for 1 hour in a mixer. Clarify using Carrez 1 (5.2) and II (5.3) reagents as described in 6.1; this time however using four times the quantities of each reagent. Bring volume up to 1 litre mark with 80% aqueous ethanol (v/v). Mix thoroughly and filter. Eliminate the ethanol as described in 6.1. If there is no dextrinised starch, bring volume up to the mark (1 litre) with distilled water.

9.2 In the case of molasses and feed materials that are rich in sugar and almost starch-free (e.g. carobs, dried beetroot cossettes etc.), weigh 5 g, place in a 250 ml volumetric flask, add 200 ml of distilled water and mix in a mixer for 1 hour, or more if necessary. Clarify using Carrez I (5.2) and II (5.3) reagents as described in 6.1. Bring volume up to the mark with cold distilled water, mix thoroughly and filter. In order to determine the amount of total sugars, continue as described in 6.3.

9.3 Because lactose is also a reducing sugar, this method also captures the content of lactose. In the case of milk replacers or other feeds containing milk or milk products it must be noted that the content of reducing sugars also includes lactose.

9.4 If the reducing sugar content in the 25 ml portion is too high, a smaller aliquot of the solution should be taken. The total volume should be adjusted to 25 ml by addition distilled water.

10. Interferences, troubleshooting and safety

In order to prevent foaming it is advisable to add (irrespective of the volume) approximately 1 ml of 3-methylbutan-l-ol (3.14) before boiling with the Luff-Schoorl reagent.

11. References

Commission Regulation (EC) No 152/2009. 27 Jan 2009. *Laying down the methods of sampling and analysis for the official control of feed.* Annex III, J, *Official Journal of the European Union* L54/1 from 26/02/2009.

GROSS ENERGY

1. Principle

Gross energy is measured by combustion of a sample in an excess of oxygen in a bomb calorimeter under standardized conditions. The gross calorific value is calculated from the temperature rise of the water in the calorimeter vessel and the mean effective heat capacity of the calorimeter.

2. Scope

This method is applicable to all feeds (see remark 9.1).

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Balance, accurate to 0.1 g, capacity 5000 g.
4.2 Balance, analytical accurate to 0.1 mg.
4.3 Bomb calorimeter, automatic adiabatic.
4.4 Oxygen fill unit.
4.5 Titration unit (optional).

5. Reagents

5.1 Cotton thread.
5.2 Platinum wire.
5.3 Benzoic acid (combustion tablets or standard grade crystals).
5.4 Phenolphthalein indicator (0.1% in 95% ethanol).
5.5 Barium hydroxide, 0.1 N (17.13 g/litre).
5.6 Sodium carbonate, 0.1 N (14.2 g/litre).
5.7 HCl, 0.1 N (place 500 ml in a 1-L flask, add 8.73 ml concentrated HCl, dilute to 1 litre with distilled water).
5.8 Methyl orange.

6. Procedure

Before starting the procedure also check the instructions of the manufacturer.

This procedure contains three parts. The first part (6.1) describes the determination of the hydrothermal equivalent of the bomb calorimeter and has to be performed annually or

if something changes within the equipment, such as use of a new bomb vessel or of a vessel after maintenance. The second part (6.2) describes the procedure to measure samples. The third part (6.3) describes the determination of the acidity, which is a correction factor for the observed values. The laboratory should decide to use this correction based on their accuracy requirements (see remark 9.2).

6.1 Determine the hydrothermal equivalent (water value) of the bomb calorimeter

6.1.1 Use a sample of known calorific value (benzoic acid combustion tablet) to determine the hydrothermal equivalent (Joules/degree rise in temperature) of the bomb, bucket, and water. Calculate the temperature rise using the above procedure. Make four determinations and calculate the mean value (this value should not change unless parts of the bomb are replaced).

6.1.2 Dry benzoic acid (5.3) at 105 ± 2 °C overnight and cool in a desiccator. Weigh 1 g of dry benzoic acid crystals, make into a tablet and reweigh. Determine the temperature rise from the combustion of benzoic acid in the bomb calorimeter.

6.1.3 For calculations required to determine the hydrothermal equivalent including corrections for the cotton thread, platinum wire, and the heat liberated in the formation of the acid (see 7. Calculation).

6.2 Measuring samples

6.2.1 Weigh approximately 1 g of sample to the nearest 0.1 mg (W) (see remark 9.1) and place in the combustion cup.

6.2.2 Attach 10 cm platinum wire (5.2) between the electrodes of the bomb and set the combustion crucible with the sample in place in the loop electrode.

6.2.3 Tie 6.5 cm of cotton thread (5.1) at the middle of the wire. Adjust the thread so that it touches the sample.

6.2.4 Assemble the bomb, tighten the screw cap, close the pressure release valve and fill with oxygen to 25–30 atmospheres.

6.2.5 Weigh 2000 g distilled water in the calorimeter bucket and place in the calorimeter. Set the bomb in the bucket and attach the clip terminal.

6.2.6 Close the cover, lower the thermometers and start the water circulating motor. Remove the cap from the jacket cover and fill the cover with water until it runs out the drain hose.

6.2.7 Adjust the temperature of the water in the outer jacket to approximately equal that of the calorimeter by adding hot or cold water, and allow 1 minute to attain equilibrium.

6.2.8 Read and record the initial temperature to the nearest 0.002 °C and ignite the sample. Wait until temperature rise is maximum, read and record final temperature. This may also be done automatically by the apparatus.

6.2.9 Open the calorimeter, take the bomb from the bucket, release the residual pressure of the bomb and open.

6.3 Determination of acidity (see remark 9.2)

6.3.1 After burning is complete, remove the bomb, release the pressure, and open. Rinse all inner bomb surfaces with a stream of distilled water, collect all washings in a clean beaker and make washings up to 100 ml.

6.3.2 Filter and boil to remove carbon dioxide.

6.3.3 Titrate hot filtrate to phenolphthalein (5.4) end point with 0.1 N barium hydroxide (5.5) (A).

6.3.4 Add 20 ml of 0.1 N sodium carbonate (5.6) (B), filter the precipitate and wash with distilled water.

6.3.5 Cool and titrate with 0.1 N HCl (5.7) (C) using methyl orange (5.8) as indicator.

7. Calculation

7.1 The formula used for calculation of hydrothermal equivalent (He) of the bomb is:

$$He = \frac{W \times A - (L \times C) - 14}{Tf - Ti}$$

where,

He = hydrothermal equivalent (J/°C),
W = weight of benzoic acid sample (g),
A = joules per gram benzoic acid, i.e. 26442 J/g,
L = weight of cotton thread (g),
C = joules per g cotton, i.e. 17500 J/g,
14 = correction for acid formation (J),
Tf = final temperature, and
Ti = initial temperature.

For cotton thread, values given by suppliers can be used.

Correction for combustion of platinum wire is very small and can be neglected.

Value for acid formation is also small and is fixed to 14 J. This value can also be measured by following procedure 6.3 with 1 ml of distilled water and calculate the correction value as described in 7.3.

7.2 The formula used for calculation of the gross energy (GE) content of samples is:

$$GE\ (kJ/g) = \frac{(Tf - Ti) \times He}{W}$$

where,

Tf = final temperature (°C),
Ti = initial temperature (°C),
W = weight of sample (g), and
He = hydrothermal equivalent (J/°C)

Express the results in kJ/g.

7.3 Calculation of the acid correction factor

Calculation of the acid factor is done by:

Nitric acid correction (J) = 6.0 (B – C)

$$\text{Sulphuric acid correction (J/W)} = \frac{15.1\ (A - (B - C))}{W}$$

The corrections are 94.6 J for 0.01 g (1%) of sulphur in the fuel and 6 J/ml of 0.1 N nitric acid formed.
This factor should be used instead of the fixed value of 14 J.

8. Quality Control

Benzoic acid and a control sample should be run with each day's batch.

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range. Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall be < 0.5% compared to the higher result.

9. Remarks

9.1 For liquid samples, approximately 5–10 g should be weighed into a bag and dried in a vacuum oven or freeze dried before analysis.

9.2 Variation in acid formation has only a very small effect on the values found. Therefore, most laboratories use a fixed value (i.e. 14 J) for acid correction.

10. References

Hill, W.H., Seals, J., and Montiegel, E. 1958. Destruction of animal and vegetable tissue by combustion in a Parr oxygen bomb. *Am. Ind. Hyg. J.* 19: 378–81.

ISO 9831. 1998. *Animal feeding stuffs, animal products, and faeces or urine – Determination of gross calorific value – Bomb calorimeter method.* Geneva, Switzerland.

Parr Manual 120. 1948. *Oxygen bomb calorimetry and oxygen bomb combustion methods.* Parr Instrument Company, Moline, IL, USA.

VOLATILE FATTY ACIDS (VFA) IN SILAGE – GAS CHROMATOGRAPHY

1. Principle

A water extract from silage is acidified and centrifuged. The alcohols and volatile fatty acids (VFA) are separated in the chromatography column depending on their molecular weight and then detected, identified, amplified, and areas integrated.

2. Scope

The procedure for determination of alcohols and volatile fatty acids is only applicable to silages.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility

of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1. Gas-liquid chromatograph provided with a flame ionization detector.
4.2. Capillary column EC-1000 (L = 30 m, ID = 0.53 mm) with a stationary phase of acid modified polyethylene glycol (thickness = 1.20 µm) (see remark 9.1).
4.3. Integrator (software or chart recorder).
4.4. Centrifuge.
4.5. Vortex mixer.
4.6 Balance accurate to 0.1 g.

5. Reagents

5.1. Oxalic acid dehydrate (solvent); make solutions of 0.12 M and 0.03 M.
5.2. Iso-butanol 99.9% (internal standard for alcohols); make solution of 10 µmol/ml.
5.3. Iso-caproic acid 99.0% (internal standard for VFA); make solution of 10 µmol/ml.
5.4. Standard mixture: pipette 12.5 ml oxalic acid solution (0.12 M) in a graduated flask of 50 ml and add 65.40 µl acetic acid, 10.60 µl propionic acid, 5.02 µl iso-butyric acid, 13.00 µl butyric acid, 2.48 µl iso-valeric acid, 2.98 µl valeric acid, 4.00 µl methanol, 72.40 µl ethanol, 5.74 µl propanol and 1.98 µl butanol and make up to the mark with distilled water.
5.5. Make blank solvent (Bs): 5 ml of oxalic acid solution (0.03 M).
5.6. Make blank internal standard (Bis): 4 ml of oxalic acid solution (0.03 M) + 0.5 ml isobutanol solution and 0.5 ml iso-caproic solution.
5.7. Make calibration standards: 3.5 – 3.0 – 2.5 – 2.0 – 1.5 ml of oxalic acid solution (0.03 M) + 0.5 ml isobutanol solution and 0.5 ml isocaproic solution + 0.5 – 1.0 – 1.5 – 2.0 – 2.5 ml of the standard mixture (5.4).
5.8. Make a positive control by adding 0.2 ml isobutanol solution (5.2) and 0.2 ml isocaproic solution (5.3) to 1.6 ml of the standard mixture (5.4).

6. Procedure

6.1. Weigh 100 g of silage in a 1-L volumetric flask and add distilled water up to the mark. Let the silage soak for 16 hours (overnight) in a refrigerator (2–8 ˚C). Filter through a filter paper.
6.2. Analyse immediately or put the extract in the freezer. In the latter case, take the silage extract out of the freezer and transfer to a refrigerator (2–8 ˚C) a day before analysis.
6.3. Pipette subsequently 2.0 ml of 0.3 M oxalic acid solution (5.1), 0.5 ml isobutanol solution (5.2), 0.5 ml iso-caproic solution (5.3) and 2 ml of the silage extract into a 10 ml tube. Mix using a vortex (4.5). Centrifuge (4.4) at 2600 *g* for 5 minutes. Fill a vial with 1.25 ml and flush with nitrogen.
6.4. Set the gas chromatograph (4.1) according to the manufacturer's instructions; among others: helium gas flow at 7.2 ml/minute, injection Port at 220 °C, column at 200 °C and detector at 220 °C.

6.5. A series starts with 2 blank solvents (5.5), then a blank internal standard (5.6), then a positive control (5.8) and finally a blank internal standard (5.6). After each 10 samples a control in-between containing subsequently blank internal standard (5.6), a positive control (5.8) and finally a blank internal standard (5.6) are analysed. The series ends with the analysis of subsequently blank internal standard (5.6), a positive control (5.8) and finally a blank internal standard (5.6).

6.6. Inject 1 µl of the prepared sample by means of a split/splitless injector (split 1/10) on the wide bore capillary column (4.2). The volatile components are separated by means of the carrier gas helium and the medium polar stationary phase (acid modified polyethylene glycol) using a temperature gradient (80 °C for 5 minutes, 10 °C/minute up to 200 °C and 200 °C for 8 minutes), and are detected by the flame ionisation detector.

NOTE: Between two injections the oxalic acid solution (0.03 M) is used to rinse the column.

7. Calculation

The identification and quantification of the components is based on a multilevel internal standard calibration. For each component the relative retention time and the surface under the peak are measured.

For each component a calibration curve is established. From this the concentration of the component (µmol/ml) in the sample is derived taking into account the dilution factor. The concentration, % in the silage, is calculated by multiplying by the molar weight.

8. Quality control

Frequent inclusion of the blank standard is required between the samples to prevent carry-over effect to the analysis of subsequent samples.

Frequent analysis of the blank internal standard is required to evaluate chromatographic conditions.

Frequent analysis of a control sample is required to control the identification and quantification of the components.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall be < 5% of the average value.

9. Remarks

9.1 Other types of GC columns that are capable to separate alcohols and volatile fatty acids can be used.

10. References

Block, H.-J. & Weissbach, F. 1982. Zur gaschromatografiphischen Bestimmung flüchtiger Fettsäuren in Silagen mit innerem Standard. *Arch. Tierennährung* 32 (9): 693–702.

Fussell, R.J. & McCalley, D.V. 1987. Determination of volatile fatty acids (C_2 - C_5) and lactic acid in silage by gas chromatography. *Analyst* 112: 1213–1216.

Jouany, J.P. 1981. Dosage des acides gras volatils et des alcools dans les ensilages par chromatographie en phase gazeuse. *Bull. Techn. C.R.Z.V. Theix, I.N.R.A*. 46: 63–66.

Jouany, J.P. 1982. Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents. *Sciences des aliments* 2: 131–144.

Ottenstein, D.M. & Bartley, D.A. 1971. Improved gas chromatography separation of free acids C_2 - C_5 in dilute solution. *Anal. Chem.* 43 (7): 952–955.

LACTIC ACID IN SILAGES – ENZYMATIC METHOD

1. Principle

In an aqueous extract of the silage, lactic acid (D- and L-lactate) is oxidised by nicotinamide adenine dinucleotide (NAD^+) to pyruvate in the presence of lactate dehydrogenase (LDH). A second reaction is catalyzed by glutamate pyruvate transaminase (GPT) to form NADH. This is measured using a spectrophotometer at 340 nm, which is stoichiometrically equivalent to the lactic acid present.

2. Scope

The procedure for determining lactic acid is applicable only to silages.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1. Centrifuge.
4.2. Spectrophotometer.
4.3 Balance accurate to 0.1 g.

5. Reagents

5.1. Enzyme kit for determination of D- and L-lactic acid.
5.2. Lactate (D+L) standard.

6. Procedure

6.1. Place 100 g of silage in a 1-L volumetric flask and add distilled water to the mark. Let it soak for 16 hours (overnight) in a refrigerator (4–8 ˚C). Filter through a filter paper.
6.2. Analyse immediately or place the extract in the freezer. In the latter case, take the silage extract out of the freezer and place in the refrigerator (4–8 ˚C) a day before analysis.
6.3. Pipette 5 ml of the sample into a 10 ml plastic tube and centrifuge (4.1) at 3000 *g* for 5 minutes.
6.4. All solutions from the enzyme kit (5.1) are ready to use, with the exception of the NAD-solution.
6.5. Transfer into 2 ml quartz cuvettes the buffer solution, NAD, GPT, distilled water and the sample in the quantities according to the instructions of the enzyme kit. Include also a blank and a standard solution (5.2).
6.6. Place the cuvettes in the spectrophotometer (4.2) and read the absorbance after exactly 5 minutes (A1).

6.7. Add the D-LDH solution into the cuvettes, place in the spectrophotometer and read the absorbance after exactly 40 minutes (A2).

6.8. Add the L-LDH solution to the cuvettes, place in the spectrophotometer and read the absorption after exactly 40 minutes (A3).

6.9. If A3 is > 1, the extract has to be further diluted.

7. Calculation

D-lactic acid in g/l = (A2 – A1) x DF x 0.3204

L-lactic acid in g/l = (A3 – A2) x DF x 0.3232

Correct the results for the dry matter (DM) content of the sample:

D-/L-lactic acid x (1000 – DM)/1000

where,

A1 = absorbance after 5 minutes,

A2 = absorbance after addition of D-LDH,

A3 = absorbance after addition of L-LDH,

DF = dilution factor, and

DM = dry matter content in %.

As the extract is obtained by diluting a 100 g sample to 1 litre, the result is in % of the silage.

8. Quality Control

In each run the standard solution of D- and L-lactate should be analysed to control precision.

Samples should be analysed in duplicate and differences between duplicates should not be greater than 2.8 times the intra-laboratory reproducibility. The latter is calculated as the variation coefficient (relative standard deviation = SD in % of the mean) of the results on 1 or 2 silage samples analysed on different days and eventually by different laboratory analysts.

9. Interferences and troubleshooting

9.1. See instructions with the enzyme kit

10. References

Gawehn, K. 1984. D-Lactic acid/L-Lactic acid. In: *Methods of Enzymatic Analysis* (Bergmeyer H.U., ed.) 3rd ed., vol. VI, pp. 588–592. Verlag Chemie, Weinheim, Deerfield Beach/Florida, Basel.

Noll, F. 1966. Methode zur quantitativen Bestimmung van L(+)-Lactat mittels Lactat-Dehydrogenase und Glutamat-Pyruvat-Transaminase, *Biochem*. Z. 346: 41–49.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex III, K, *Official Journal of the European Union* L54/1 from 26/02/2009 – Determination of Lactose using Titration.

UREA – SPECTROPHOTOMETRIC METHOD

1. Principle

The sample (feed or feed ingredient) is suspended in distilled water with a clarifying agent. The urea content is determined using a spectrophotometer after addition of 4-dimethyl-aminobenzaldehyde (4-DMAB).

2. Scope

The method is suitable for feeds and forages. Some feeds have added urea as a supplemental source of nitrogen. This method describes determination of urea, ammonia content is not included (see remark 9.4).

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1. Mixer (tumbler), approximately 35 to 40 rpm.
4.2. Test tubes, 160 mm × 16 mm with ground-glass stoppers.
4.3. Spectrophotometer.

5. Reagents

5.1 Solution of 4-dimethylaminobenzaldehyde (4-DMAB)
Dissolve 1.6 g of 4-DMAB in 100 ml of 96% aqueous ethanol and add 10 ml of HCl (37% HCl or ρ_{20} = 1.19 g/ml). This reagent can be kept for a maximum period of two weeks.

5.2 Carrez solution I
Dissolve 21.9 g of zinc acetate, $Zn(CH_3COO)_2.2\ H_2O$ and 3 g of glacial acetic acid in distilled water. Make up to 100 ml with distilled water.

5.3 Carrez solution II
Dissolve 10.6 g of potassium ferrocyanide, $K\ Fe(CN)_6.\ 3\ H_2O$ in distilled water. Make up to 100 ml with distilled water.

5.4 Activated carbon which does not absorb urea (check this before using).

5.5 Urea, 0.1% solution (w/v).

6. Procedure

6.1 Sample Analysis

6.1.1 Weigh 2 g of the sample to the nearest mg and place with 1 g of active carbon (5.4) in a 500 ml volumetric flask.

6.1.2 Add 400 ml of distilled water and 5 ml of Carrez solution I (5.2), mix for approximately 30 seconds and add 5 ml of Carrez solution II (5.3).

6.1.3 Mix for 30 minutes and make up to the mark with distilled water, mix and filter.

6.1.4 Transfer 5 ml of the transparent colourless filtrate to a test tube with ground-glass stopper, add 5 ml of 4-DMAB solution (5.1) and mix.

6.1.5 Place the tubes in a water bath at 20 ± 4 °C.

6.1.6 After 15 minutes measure the absorbance of the sample solution using a spectrophotometer at 420 nm. Compare with the corresponding blank solution (containing 5 ml of 4-DMAB and 5 ml of distilled water free from urea).

6.2 Calibration curve

6.2.1 Transfer 1, 2, 4, 5 and 10 ml of the urea solution (5.5) into 100 ml volumetric flasks and make up to the mark with distilled water.

6.2.2 Take an aliquot of 5 ml from each solution, add 5 ml of 4-DMAB solution (5.1), mix well and measure the absorbance against a blank containing 5 ml of 4-DMAB and 5 ml of water free from urea.

6.2.3 Measure absorbance at 420 nm using a spectrophotometer and plot the calibration curve.

7. Calculation

Concentration of urea in the sample solution is determined from the linear regression of the calibration curve (6.2.3).

$$c_s = \frac{A_s - b}{m}$$

where,

c_s = urea concentration of the sample solution in mg/100 ml,
A_s = absorbance value of the sample solution,
B = y-intercept of the regression line, and
m = slope of the regression line.

Urea content of the sample considering the conditions as mentioned in 6.1 (weight of 2 g in 500 ml solvent) is calculated as:

$$urea\ [\%] = c_s \cdot 0.25$$

Urea content of the sample in general is calculated as:

$$urea\ [\%] = \frac{c_s \cdot V \cdot F}{w \cdot 1000}$$

where,

V = volume of the sample solution in ml,
F = the dilution factor, and
w = the sample weight in g.

8. Quality Control

A control sample containing 2% urea should be run with each batch. This sample can be prepared by mixing 2 g urea in 98 g of a dry urea-free feed that is similar to the sample being analysed. Recovery should be 90 to 110%, otherwise the series must be repeated.

NOTE: The control feed sample containing urea should be kept in a dessicator. An old sample (> one month) should not be used.

The difference between duplicates should be lower than 5% relative to the higher value.

9. Remarks

9.1 If urea concentration exceeds 3%, reduce the sample to 1 g or dilute the original solution so that there is no more than 50 mg of urea in 500 ml.

9.2. If urea concentration is low, increase the sample amount (as long as the filtrate remains transparent and colourless).

9.3 The absorbance strongly depends on the temperature. It is therefore recommended to carry out the measurements for establishing the calibration curve and for the test samples at the same time.

9.4 Ruminant animals can hydrolyze urea into ammonia, thereby using the ammonia to convert into a protein source. However, if urea is added in excess in feeds, it can be toxic to the animal.

10. Troubleshooting

10.1 Since many amino acids under the conditions described above exhibit absorption maximum at about 415 nm, the urea determination at 420 nm is considerably compromised. At 435 nm the absorbance produced by amino acids is substantially lower and that by urea is only slightly lower than at 420 nm. As most feed samples contain simple nitrogenous compounds such as amino acids, it is generally recommended to perform the measurements for the test samples and the calibration curve at 435 nm.

11. References

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Augustin, T. & Eckstein, M. 2010. Report for ring test urea Nr. 387Q. *Landesbetrieb Hessisches Landeslabor*. Kassel, Am Versuchsfeld 13, 34128 Kassel.

ELEMENTS – AAS

1. Principle

Feed material is ashed in a muffle furnace and the resulting ash is dissolved in hydrochloric acid. After filtration and appropriate dilution trace elements are determined by an atomic absorption spectrophotometer.

2. Scope

The described procedure is applicable for the determination of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), potassium (K), sodium (Na) and zinc (Zn) in all animal feedstuffs.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1. Analytical balance, accurate to 0.1 mg.
4.2. Electric muffle furnace, capable of being maintained at 550 ± 20 °C.
4.3 Hot plate.
4.4 Atomic Absorption Spectrophotometer (AAS).
4.5 Glassware must be of resistant borosilicate type and it is recommended to use apparatus which is reserved exclusively for trace element determinations.

5. Reagents

Use reagent grade chemicals and deionized water unless otherwise specified.

5.1 Hydrochloric acid, c = 12 M.
5.2 Hydrochloric acid, c = 6 M.
5.3 Hydrochloric acid, c = 0.6 M.
5.4 Lanthanum nitrate solution
Dissolve 133 g of $La(NO_3)_3.6\ H_2O$ in 1 litre of deionised water
5.5 Caesium chloride solution
Dissolve 100 g of CsCl in 1 litre of deionised water
5.6 Stock solution of Cu, Fe, Mn and Zn
Mix 100 ml of deionised water and 125 ml of hydrochloric acid (12 M) in a 1-L volumetric flask. Weigh the following:
392.9 mg of copper(II) sulfate pentahydrate, $CuSO_4.5\ H_2O$
702.2 mg of ammonium iron(II) sulfate hexahaydrate, $(NH_4)_2SO_4.FeSO_4.6\ H_2O$
307.7 mg of manganese sulfate monohydrate, $MnSO_4.H_2O$
439.8 mg of zinc sulfate heptahydrate, $ZnSO_4.7\ H_2O$
Transfer the weighed salts to the volumetric flask and dissolve. Dilute to the mark with deionised water.
5.7 Standard solution of Cu, Fe, Mn and Zn
Dilute 20 ml of the stock solution with deionised water in a 1-L volumetric flask and dilute to the mark with deionised water.
5.8 Standard solution of Ca, K, Mg and Na
Dilute 25 ml of stock solution (5.8) with dilute hydrochloric acid (5.3) to 250 ml in a volumetric flask. The contents of Ca, K and Na are each 100 µg/ml, the content of Mg is 20 µg/ml. Prepare the solution fresh for the week of use and store it in a polyethylene bottle.
5.9 Lanthanum-caesium blank solution
Add 5 ml of lanthanum nitrate solution (5.4), 5 ml of caesium chloride solution (5.5) and 5 ml of hydrochloric acid (5.3) to a 100 ml volumetric flask. Dilute to the mark with deionised water.

6. Procedure

6.1 Sample preparation

6.1.1 Place 5–10 g of sample weighed to the nearest 0.2 mg in a quartz or platinum crucible, dry in an oven at 105 ± 2 °C and introduce the crucible into the cold muffle furnace (4.2).

6.1.2 Close the furnace and gradually raise the temperature to 550 ± 20 °C over approximately 90 minutes. Maintain this temperature for 4 to 16 hours (e.g. overnight) to remove carbonaceous material and then open the furnace and allow to cool.

6.1.3 Moisten the ash with deionised water and transfer it into a 250 ml beaker. Wash the crucible with a total of approximately 5 ml of hydrochloric acid (5.1). *NOTE:* Add the acid slowly and carefully to the beaker (there may be a vigorous reaction due to CO_2 formation).

6.1.4 Add hydrochloric acid (5.1) drop-wise with agitation until all effervescence has stopped.

6.1.5 Evaporate to dryness, occasionally stirring with a glass rod.

6.1.6 Add 15 ml of 6 M hydrochloric acid (5.2) to the residue followed by about 120 ml of deionised water. Stir with the glass rod, which should be left in the beaker, and cover the beaker with a watch-glass.

6.1.7 Bring gently to the boil and maintain at boiling point until no more ash can be seen to dissolve.

6.1.8 Filter on ash-free filter paper and collect the filtrate in a 250 ml volumetric flask.

6.1.9 Wash the beaker and filter with 5 ml of hot 6 M hydrochloric acid (5.2) and twice with boiling water.

6.1.10 Make up to the mark with deionised water (HCl concentration approximately 0.5 M).

6.1.11 If the residue on the filter appears black (carbon), put it back in the furnace and ash again at 450 to 475 °C. This ashing requires about 3–5 hours and is complete when the ash appears white or nearly white.

6.1.12 Dissolve the residue with approximately 2 ml of hydrochloric acid (5.1), evaporate to dryness and add 5 ml of 6 M hydrochloric acid (5.2).

6.1.13 Heat, filter the solution into the volumetric flask and make up to the mark with deionised water (HCl concentration approximately 0.5 M).

NOTE: Other methods of digestion may be used provided they have been demonstrated to have similar results (e.g. microwave digestion).

If the sample contains no organic matter (e.g. mineral feed), prior ashing is unnecessary. Proceed as described in point 6.1 number 3.

6.2 Spectrophotometric determination of Fe, Cu, Mn and Zn

6.2.1 Measuring conditions

Adjust the AAS (4.4) in accordance with the manufacturer's instructions and optimise the response of the instrument using an oxidising air-acetylene flame at the following wavelengths:

F : 248.3 nm
Cu : 324.8 nm
Mn : 279.5 nm
Zn : 213.8 nm

6.2.2 Preparation of calibration curves

1. Prepare a series of appropriate calibration solutions by diluting the standard solution (5.7) with dilute hydrochloric acid (5.3).

2. Measure the absorbance of the hydrochloric acid (5.3) and the absorbance of the calibration solutions and subtract the absorbance measured for the hydrochloric acid.

3. Draw a calibration curve by plotting the corrected absorbances against the respective contents of Cu, Fe, Mn and Zn.

6.2.3 Measurement of test solution

1. Measure parallel to the calibration solutions, under identical conditions, the absorbance of the test solution and the blank solution. Subtract the latter absorbance from the first absorbance.

2. If necessary, dilute an aliquot of the test solution and blank solution with dilute hydrochloric acid (5.3) to obtain an absorbance in the linear part of the calibration curve.

6.3 Spectrophotometric determination of Ca, Mg, K and Na

6.3.1 Measuring conditions

Adjust the AAS (4.4) in accordance with the manufacturer's instructions and optimise the response of the instrument using an oxidising air-acetylene flame at the following wavelengths:

Ca : 422.6 nm
Mg : 285.2 nm
K : 766.5 nm
Na : 589.6 nm

6.3.2 Preparation of calibration curves

1. Dilute the standard solution (5.8) with deionised water. To 100 ml of the diluted standard solution add 5 ml of lanthanum nitrate solution (5.4), 5 ml of caesium chloride solution (5.5) and 5 ml of hydrochloric acid (5.2). Choose the dilution so that appropriate calibration solutions are obtained.

2. Measure the absorbance of the lanthanum-caesium blank solution (5.9).

3. Measure the absorbance of the calibration solutions and subtract the absorbance measured for the lanthanum/caesium blank solution (5.9).

4. Draw a calibration curve by plotting the corrected absorbance's against the respective contents of Ca, Mg, K and Na.

6.3.3 Measurement of test solution

1. Dilute an aliquot of the test solution and blank solution with deionised water. To 100 ml of diluted standard solution add 5 ml of lanthanum nitrate solution (5.4), 5 ml of caesium chloride solution (5.5) and 5 ml of hydrochloric acid (5.2).

2. Measure parallel to the calibration solutions, under identical conditions, the absorbance of the diluted test solution and the diluted blank solution. Subtract the latter absorbance from the first absorbance.

3. If necessary, dilute an aliquot of the test solution and blank solution with lanthanum/caesium blank solution (5.9) to obtain an absorbance in the linear part of the calibration curve.

7. Calculation

Using a calibration curve (6.2.2 and 6.3.2), calculate the trace element concentration in the solution:

$$c_s = \frac{A_s - b}{m}$$

where,

c_s = element concentration of the sample solution [µg/ml],
A_s = Absorbance value of the sample solution,
b = y-intercept of the regression line, and
m = slope of the regression line.

Element content of the sample in mg/kg considering dilution steps is calculated as:

$$element\ [mg/kg] = \frac{c_s \cdot v \cdot F}{w}$$

where,

v = volume of the sample solution [ml],
F = dilution factor, and
w = sample weight [g].

Express the result in milligrams of trace element per kilogram of sample (ppm), and for the macro elements in grams per kilogram (g/kg).

8. Quality Control

- Duplicates of a working control should be run with each set and compared to limits on the established control chart. A spiked Blank at a level of the medium concentration of the calibration curve must result in a recovery between 80 and 120%.
- One blank should be run with each set. A 10 ml aliquot will be taken and treated as a sample. Calculated concentration must be < 0.5 ppm.
- Immediately following creation of the calibration curve, a 0 ppm reference standard should be read as a sample. The calculated concentration of the reading must be < 0.1 ppm.
- Immediately following the reading of the 0 ppm reference standard, the 10 ppm reference standard should be read as a sample. The reading must be within 2.5% of 10 ppm (9.75–10.25 ppm).
- If the blank and/or 10 ppm standard fail, then the instrument must be recalibrated by creating a completely new calibration line using all reference standards. Immediately following the recalibration, the 0 ppm standard and 10 ppm standard must be retested and must meet the acceptance criteria stated above.

- The 0 and 10 ppm standards should be run at the beginning and the end of each run and after every 10 samples.

9. Interferences, troubleshooting and safety

9.1. Lanthanum ionization buffer must be added to each sample. Its presence can be detected by a green flame during analysis. Low results will result if ionization buffer is omitted.

9.2. During the hydrochloric acid fuming step, some digests suddenly change appearance (colour, particle shape), forming less soluble components. It may be necessary to re-digest a new sample just to the point when the hydrochloric acid begins to fume.

10. References

AOAC 968.08. 2000. *Minerals in animal feed and pet food, atomic absorption spectrophotometric method.* Gaithersburg, MD, USA.

AOAC 965.09. 2000. *Nutrients (minor) in fertilizers, atomic absorption spectrophotometric method.* Gaithersburg, MD, USA.

Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex IV, C, *Official Journal of the European Union* L54/1 from 26/02/2009.

ISO 6869. 2000. *Animal feeding stuffs – Determination of the contents of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc – Method using atomic absorption spectrometry.* Geneva, Switzerland.

CALCIUM – SPECTROPHOTOMETRIC METHOD

1. Principle

The sample is ignited at 550 °C to burn all organic material. The remaining minerals are digested in 6 M HCl to release calcium, which is then determined using a spectrophotometric assay based on reaction of calcium with o-cresolpthalein complexone (CPC) in alkaline solution. Magnesium is masked by 8-hydroxyquinoline.

2. Scope

This method is applicable for feed, food, digesta and faeces. This method is not applicable for mineral-mix feeds.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate at 0.1 mg.

4.2 Furnace-proof beakers e.g. Pyrex beakers.

4.3 Muffle furnace, capable of being maintained at 550 ± 20 ˚C.
4.4 Hot plate.
4.5 Spectrophotometer.

5. Reagents

5.1 Hydrochloric acid, 6 M.
5.2 Standard solution of calcium.
5.3 Test kit for calcium, e.g. Ref No.11489216 216 from Roche.
Kits from other manufacturers based on the CPC method can also be used.

6. Procedure

6.1 Sample preparation

6.1.1 Weigh approximately 1 g to the nearest 0.2 mg (W) in a beaker (4.2) and place in cold Muffle furnace (4.3).

6.1.2 Close the furnace and gradually raise the temperature to 550 °C over about 90 minutes. Maintain this temperature for 16 hours (e.g. overnight) to remove carbonaceous material and then open the furnace and allow to cool (see remark 9.1).

6.1.3 Add 10 ml 6 M hydrochloric acid (5.1) to each beaker and place on a preheated hot plate (approximately 250 ˚C), cover the beakers with a glass plate, digest for 20 minutes.

6.1.4 Allow the beakers to cool and remove from the hot plate.

6.1.5 Transfer quantitatively the content of the beakers to a 25 ml volumetric flask, make up to the mark with distilled water and mix well.

6.1.6 Measure calcium in the solutions (6.1.5) and standards (5.2) by the CPC method by following the instructions of the manufacturer of the test kit (5.3), measure the absorbance at 578 nm (see remark 9.2).

7. Calculation

Calculate the calcium content in the measured solution by linear regression.
Percent of calcium is calculated as:

$$\% \text{ Calcium} = (C \times V \times DF) / (W \times 10)$$

where,

C = concentration calcium in measure solution (mg/litre),
V = volume of solution (in litres, i.e. 0.025 (L)),
DF = dilution factor (normally, i.e. 1),
W = weight of the sample (g), and
10 = factor to convert g/kg to %.

8. Quality Control

High purity calcium containing salt can be used as a control standard, average 15–20 replicates and allow ± 2 SD as an acceptable range.

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the

samples to be analysed. Take 3–4 kg of the chosen QC sample, grind and pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range. Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample shall be < 5% of the average value.

9. Remarks

9.1 Other methods of digestion may be used provided they have been validated to give similar results (e.g. microwave digestion).

9.2 Determination can be performed automatically by using an auto analyser. This will improve the precision and efficiency of the method.

10. References

AOAC 968.08D. 2000. *Acid digestion*. Gaithersburg, MD, USA.

Tietz, N.W. 1995. Calcium determination. *Clinical Guide to laboratory Tests, 3 Auflage*. Philadelphia, Pa: WB Saunders Company.

Gosling, P. 1986. Analytical reviews in clinical biochemistry: Calcium measurement. *Ann Clin Biochem* 23: 146–156.

PHOSPHORUS – SPECTROPHOTOMETRIC METHOD

1. Principle

Feed material is ashed following digestion in hydrochloric acid. Molybdovanadate reagent is added which results in a characteristic yellow color after reacting with phosphorus, which is measured spectrophotometrically.

2. Scope

The method is applicable to animal feed stuffs with a phosphorus content of < 50 g/kg. For samples with higher phosphorus content a gravimetric method is advised i.e. quinoline phosphomolybdate.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per the prescribed method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.

4.2 Furnace-proof beakers e.g. Pyrex beakers.

4.3 Muffle furnace, capable of being maintained at 550 ± 20 ˚C.

4.4 Hot plate.

4.5 UV-VIS Spectrophotometer.

5. Reagents

5.1 6 M Hydrochloric acid.

5.2 14 M Nitric acid.

5.3 Ammonium heptamolybdate solution

Dissolve 100 g of ammonium heptamolybdate tetra hydrate [$(NH_4)_6Mo_7O_{24}.4H_2O$] in hot distilled water. Add 10 ml of ammonia (NH_4OH: 14 M; $\rho(NH_4OH)$ = 0.91 g/ml) and dilute to 1 litre with distilled water.

5.4 Ammonium monovanadate solution

Dissolve 2.35 g of ammonium monovanadate (NH_4VO_3) in 400 ml of hot distilled water. Stirring constantly, slowly add 7 ml of nitric acid (5.2) and dilute to 1 litre with distilled water.

5.5 Molybdovanadate reagent

In a 1-L volumetric flask, mix 200 ml of the ammonium heptamolybdate solution (5.3), 200 ml of ammonium monovanadate solution (5.4) and 135 ml nitric acid (5.2). Dilute to the mark with distilled water. Filter if insoluble particles are present.

5.6 Phosphorus standard solution (1 mg/ml)

In a 1-L volumetric flask dissolve 4.394 g of potassium dihydrogen phosphate (KH_2PO_4) previously dried at 103 ± 2 °C for 1 hour. Dilute to the mark with distilled water.

5.7 Calibration curve.

Dilute the phosphorus standard solution to concentrations of 5, 10, 20 and 40 µg/ml. Use distilled water as a blank.

6. Procedure

6.1 Sample preparation

6.1.1 Weigh approximately 1 g to the nearest 0.2 mg (W) in a beaker (4.2) and place in cold Muffle furnace (4.3).

6.1.2 Close the furnace and gradually raise the temperature to 550 °C over approximately 90 minutes. Maintain this temperature for 16 hours (e.g. overnight) to remove carbonaceous material and then open the furnace and allow to cool (see remark 9.1).

6.1.3 Add 10 ml 6 M hydrochloric acid (5.1) to each beaker and place on a preheated hot plate (approximately 250 °C), cover the beakers with a glass plate, and digest for 20 minutes.

6.1.4 Allow the beakers to cool and remove from the hot plate.

6.1.5 Transfer the contents of the beakers quantitatively to 25 ml volumetric flasks, make up to the mark with distilled water, and mix well.

6.1.6 Let the solutions stand overnight to settle.

6.2 Measuring the phosphorus content

6.2.1 Dilute an aliquot of the solution (6.1.6) with distilled water to obtain a phosphorus content not exceeding 40 µg/ml.

6.2.2 Transfer 10 ml each of the diluted solution (6.2.1) and standard solutions (5.7) to separate test tubes. Take a test tube with 10 ml water (blank). To each tube add 10 ml molybdovanadate reagent (5.5). Mix and leave to stand for 10 minutes at 20 °C.

6.2.3 Measure the absorbance of the solution (6.2.2) at 430 nm within 45 minutes using a spectrophotometer against the blank.

7. Calculation

Calculate phosphorus content in the measured solution by linear regression.

Percentage of phosphorus is calculated as:

$$\% \text{ Phosphorus} = (C \times V \times DF) / (W \times 10)$$

where,

C = concentration phosphorus in measured solution (mg/litre),
V = volume of solution (in litres, i.e. 0.025 L),
DF = dilution factor (normally, i.e. 1),
W = weight of the sample (g), and
10 = factor to convert g/kg to %.

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a sieve of 1 mm pore size and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample should be < 5% of the average value.

9. Remarks

9.1 Other methods of digestion may be used provided they have been demonstrated to give similar results (e.g. use of different acids or microwave digestion).

10 References

Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex III, P, *Official Journal of the European Union* L54/1 from 26/02/2009.

ISO 6491. 1998. *Animal feeding stuffs – Determination of phosphorus content – Spectrometric method.* Geneva, Switzerland.

CHLORINE – TITRATION METHOD

1. Summary

Chlorides are dissolved in distilled water. If the product contains organic matter, it is clarified. The solution is slightly acidified with nitric acid and the chlorides precipitated in the form of silver chloride by means of a solution of silver nitrate. The excess silver nitrate is titrated with a solution of ammonium thiocyanate. The concentration of chloride is expressed as sodium chloride.

2. Scope

The method is applicable to animal feeds.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified

4. Equipment

4.1 Analytical balance, accurate to 0.1 mg.
4.2 Mixer.
4.3 Titration equipment.

5. Reagents

5.1 Ammonium thiocyanate solution, 0.1 M.
5.2 Silver nitrate solution, 0.1 M.
5.3 Saturated ammonium ferric sulphate $(NH_4)Fe(SO_4)_2$.
5.4 Nitric acid, ρ = 1.38 g/ml.
5.5 Diethyl ether.
5.6 Acetone.
5.7 Carrez I solution
Dissolve 21.9 g zinc acetate, $Zn(CH_3COO)_2.2\ H_2O$ and 3 g of glacial acetic acid in distilled water. Make up to 100 ml with distilled water.
5.8 Carrez II solution
Dissolve 10.6 g potassium ferrocyanide $K_4Fe(CN)_6.3\ H_2O$ in distilled water. Make up to 100 ml with distilled water.
5.9 Active carbon.

6. Procedure

6.1 Preparation of solution
According to the nature of the sample, prepare a solution as given in 6.1.1, 6.1.2 or 6.1.3.
Simultaneously carry out a blank test omitting the sample to be analysed.

Samples free from organic matter

6.1.1 Weigh maximum 10 g sample to the nearest 1 mg (W) into a 500 ml volumetric flask (see remark 9.1). Add 400 ml of distilled water to the flask and mix for 30 minutes. Make up to 500 ml with distilled water, mix thoroughly and filter.

Samples containing organic matter, excluding the products listed at 6.1.3

6.1.2 Weigh maximum 5 g sample to the nearest 1 mg (W) and 1 g active carbon (5.9) into a 500 ml volumetric flask. Add 400 ml of distilled water and 5 ml Carrez I solution (5.7) to the flask and mix for 30 minutes. Add 5 ml Carrez II solution (5.8) to the flask and mix for 30 minutes. Make up to 500 ml with distilled water, homogenise and filter.

For cooked feed, flax cakes and flour products rich in flax flour, and other products rich in mucilage or in colloidal substances;

6.1.3 Prepare the solution as described in 6.1.2 but do not filter. Remove 100 ml of the supernatant and transfer to a 200 ml volumetric flask. Make up to 200 ml with acetone (5.6), mix thoroughly and filter.

6.2 Titration

6.2.1 Using a pipette, transfer from 25 to 100 ml of the filtrate obtained (6.1) into an Erlenmeyer flask (see remark 9.2).

6.2.2 Dilute if necessary to not less than 50 ml with distilled water, add 5 ml of nitric acid (5.4), 20 ml of saturated solution of ammonium ferric sulphate (5.3) and two drops of ammonium thiocyanate solution (5.1)

6.2.3 Using a burette transfer the silver nitrate solution (5.2) into the Erlenmeyer flask (this corresponds to 5 mmol silver ions in relation to 4.23 M chloride ions when 150 mg chlorine as a maximum content is assumed, see remark 9.3) in such a way that an excess of 5 ml is obtained (see remark 9.5). Add 5 ml of diethyl ether (5.5) and shake hard to coagulate the precipitate.

6.2.4 Titrate the excess silver nitrate with the ammonium thiocyanate solution (5.1) until the reddish-brown tint has lasted for 1 minute.

7. Calculation

The amount of chlorine (X), expressed as % sodium chloride is calculated using the following formula:

$$X = 5.845 (V_1 - V_2) / W$$

where,

V_1 = volume of 0.1 M silver nitrate solution added (in ml),

V_2 = volume of 0.1 M ammonium thiocyanate solution added (in ml), and

W = weight of the sample (g).

If the blank test indicates that silver nitrate has been consumed, deduct this value from the volume ($V_1 - V_2$).

8. Quality Control

In each batch a control standard (QC sample) should be analysed. The laboratory QC sample can be made from an animal feed/forage sample that is similar in nature to the samples to be analysed. Take 3–4 kg of the chosen QC sample, grind to pass through a 1 mm pore size sieve and store in a cool, dry place. Analyse the QC sample 15–20 times, take average and allow ± 2 SD as an acceptable range.

Samples should be analysed in duplicate. The difference between the values of two parallel determinations carried out on the same sample should be < 5% of the average value.

9. Remarks

9.1 The sample should not contain more than 3 g of chlorine in the form of chlorides.

9.2 The aliquot portion must not contain more than 150 mg of chlorine.

9.3 Depending on the chloride content the volume of silver nitrate solution must be adjusted to titrate a significant volume of the thiosulphate solution.

9.4 Titration may also be carried out potentiometrically.

9.5 Available information or experience about the chlorine content of the sample should be used to obtain this excess. This excess can be checked by the volume of ammonium thiocyanate needed during the titration (6.2.4), which should be at least 4.5 ml.

10. References

Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex III, Q, *Official Journal of the European Union* L54/1 from 26/02/2009.

AFLATOXINS – HPLC METHOD

1. Principle

Aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1, AFG_2) are extracted from the feed sample with aqueous acetone. The extract is purified by immunoaffinity chromatography and the analytes are quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) with post-column derivatisation (PCD) involving bromination. The PCD is achieved with either electrochemically generated bromine or with pyridinium hydrobromide perbromide (PBPB) followed by fluorescence detection.

2. Scope

This method is for determination of aflatoxins in animal feed stuffs. It is applicable to animal feeds with a fat content of up to 50%.

The limit of quantification shall be at least 1 µg/kg for aflatoxin B_1 (signal-to-noise ratio of 6). In fact this method has been demonstrated to achieve a quantification limit of less than 0.5 µg/kg for aflatoxin B_1.

3. Responsibilities

Laboratory Analysts shall perform the analyses as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

Usual laboratory equipment and in particular the following:

4.1 Vertical- or horizontal shaker (adjustable).

4.2 Filter paper, ø 24 cm, pre-folded (e.g. Whatman 2V).

4.3 Erlenmeyer flask with screw top or glass stopper.

4.4 Glass microfiber filter paper, ø 5 cm (e.g. Whatman GF/A).

4.5 Reservoir, 75 ml with luer tip connector for immunoaffinity column.

4.6 Hand pump, 20 ml syringe with luer lock or rubber stopper for immunoaffinity column.

4.7 Volumetric glassware of 5 ml, 10 ml and 20 ml capacity, having an accuracy of at least 0.5%.

4.8 HPLC pump, suitable for flow rate of 1.000 ± 0.005 ml/min.

4.9 Injection system
Capable of total loop injection (a valve with a loop of at least 100 µl is recommended).

4.10 RP-HPLC column, e.g. SUPELCOSIL LC-18 (Supleco) or Octadecylsilane (ODS)-2 LC (Phenomenex).
Optional but recommended: pre-column.

4.11 Post column derivatisation system with PBPB (alternative to 4.12)
Second HPLC pulse-less pump, zero-dead volume T-piece, reaction tubing minimum 45 cm x 0.5 mm internal diameter PTFE (polytetrafluoroethylene) (the reaction time must be at least 4 seconds before detection).

4.12 System for derivatisation with electrochemically generated bromine (e.g. KOBRA cell), the device must be installed according to the manufacturer's the instructions.

NOTE: In order to confirm aflatoxin B1, the HPLC column has to be disconnected from the bromination device and must be connected directly to the fluorescence detector (switching-off the electrical current with the bromination device still in line is not recommended due to the possibility of remaining bromine in the cell membrane of the device).

4.13 Fluorescence detector, with a wavelength of λ = 360 nm excitation filter and a wavelength of λ > 420 nm cut-off emission filter, or equivalent
Recommended settings for adjustable detectors are Excitation = 365 nm, Emission = 435 nm, Band Width = 18 nm.

4.14 Disposable filter unit (0.45 µm).

NOTE: Prior to use it has to be verified that no aflatoxin losses occur during filtration (recovery testing) since there is a possibility that various filter materials can retain aflatoxin B_1.

4.15 Single marked pipettes, 1 ml, 2 ml, 5 ml and 10 ml capacity.

4.16 Analytical balance, accurate 0.1 mg.

4.17 Laboratory balance, accurate 0.01 g.

4.18 Calibrated microliter syringe(s) or microliter pipette(s) (20–500 µl).

4.19 Evaporator (optional; only needed for option B, section 6.3)

5. Reagents

Use only reagents of recognised analytical grade, unless otherwise specified.

All solutions are made with HPLC grade solvents and reagent grade materials unless otherwise noted.

5.1 Double distilled or deionised water.

5.2 Phosphate buffered saline, PBS, pH 7.4
PBS can be prepared from potassium chloride (0.20 g), potassium dihydrogen phosphate (0.20 g), anhydrous disodium hydrogen phosphate (1.16 g) [or disodium hydrogen phosphate.12 H_2O (2.92 g)] and sodium chloride (8.00 g) added to 900 ml double distilled water. After dissolution the pH must be adjusted to pH 7.4 (with 0.1 M HCl or 0.1 M NaOH as appropriate) and the solution made to 1.0 litre with double distilled water.
Alternatively, commercially available phosphate buffered saline tablets with equivalent properties can be used.

TABLE 3
Preparation of working calibration solutions

Working standard	Option A		Option B	
	Aliquot stock solution [µl]	Concentration [ng AFB1/ml]	Aliquot stock solution [µl]	Concentration [ng AFB1/ml]
1	20	0.050	100	0.250
2	45	0.113	225	0.563
3	70	0.175	350	0.875
4	95	0.238	475	1.188
5	120	0.300	600	1.500
6	145	0.363	725	1.813
7	170	0.425	850	2.125
8	195	0.488	975	2.438
9	220	0.550	1100	2.750

5.3 Pyridinium hydrobromide perbromide, PBPB, CAS 39416-48-3.
This reagent is not required in the case a KOBRA cell ® is used.

5.4 Potassium bromide.
This reagent is not required in the case the PBPB reagent is used.

5.5 HPLC grade acetonitrile.

5.6 HPLC grade methanol.

5.7 Acetone, pure.

5.8 HPLC grade water (double distilled or deionised water can also be used).

5.9 Extraction solvent.
acetone (5.7):double distilled water, (85:15, v/v).

5.10 Nitric acid, c(HNO3) = 4 M
This reagent is not required in the case of the PBPB reagent is used.

5.11 Immunoaffinity column
The affinity column should contain antibodies raised against aflatoxin B_1. The column should have a capacity of not less than 40 ng of aflatoxin B_1 and should give a recovery of not less than 80% for aflatoxin B_1 when applied as a standard solution in acetone:double distilled water (85:15, v/v) containing 0.25 ng of aflatoxin B_1.

5.12 HPLC mobile phase solvent A , for use with the PBPB post column reagent only:
Double distilled water (5.8):acetonitrile (5.5):methanol (5.6) solution (6:2:3, v/v/v). The ratio of solvents can be adjusted to obtain best separation.

5.13 HPLC mobile phase solvent B, for use with electrochemically generated bromine only:
Double distilled water (5.8):acetonitrile (5.5):methanol (5.6) solution (6:2:3, v/v/v)), containing 120 mg potassium bromide (5.4) and 350 µl nitric acid (5.10) per litre mobile phase. The ratio of solvents can be adjusted to obtain best separation parameters.

NOTE: The mobile phase solvent (5.12/5.13) should be degassed.

5.14 Post column reagent
For use with PBPB post column reagent only:

Dissolve 25 mg PBPB (5.3) in 500 ml double distilled H_2O. Solution can be used for up to 4 days if stored in a dark place at room temperature. This post column reagent is to be used only in combination with HPLC mobile phase solvent A (5.12) but not with HPLC mobile phase solvent B (5.13).

5.15 Toluene:acetonitrile (98:2, v/v).

5.16 Aflatoxin B_1 standard material.

Aflatoxin B_1 standard in form of crystals or a dry film for analytical purposes.

5.17 Calibration stock solutions for HPLC.

5.17.1 General

Prepare an aflatoxin B_1 (5.16) stock solution containing 50.0 ng/ml in toluene:acetonitrile (5.15).

5.17.2 Option A (see method description 6.3)

Pipette from the solution (5.16) the volumes as listed in Table 3 (Option A) into a set of 20 ml calibrated volumetric flasks. Evaporate the toluene:acetonitrile solution just to dryness under a stream of nitrogen at room temperature. To each flask, add 7 ml of methanol, let aflatoxins dissolve, then make up to the mark with double distilled water and shake well.

NOTE: Bear in mind that methanol and double distilled water are subject to volume contraction when mixed.

5.17.3 Option B (see method description 6.3)

Into a set of 20 ml calibrated volumetric flasks pipette from the solution (5.16) the volumes as listed in Table 3 (Option B). Evaporate the toluene-acetonitrile solution just to dryness under a stream of nitrogen at room temperature. To each flask, add approximately 10 ml of methanol, let aflatoxins dissolve, then make up to the mark with methanol (not with aqueous methanol) and shake well. Exactly 1 ml of this working calibration solution is then transferred into an acid washed glass vial, evaporated to dryness as described in option B under 6.3 and then re-dissolved in exactly the same volume that will be used to re-dissolve the samples prior to injection (6.3). Calculate the concentration of aflatoxin B1 in the evaporated and re-dissolved solution in ng/ml. Use these concentration values for the calculation as given in section 6.6. In this case the calibration range will remain unchanged.

6. Procedure

6.1 *Conditioning of immunoaffinity columns*

Immunoaffinity columns (5.11) should be at room temperature prior to conditioning. For conditioning follow the instructions of the manufacturer. If not stated differently, apply 10 ml of PBS (5.2) on the top of the column and let it pass at a speed of 2–3 ml/min through the column (by gravity). Make sure that a small portion (0.5 ml) of PBS remains on the column until the sample solution is applied.

6.2 *Extraction*

6.2.1 Weigh, to the nearest 0.1 g, approximately 50 g of the test portion into a 500 ml Erlenmeyer flask with a screw top or a glass stopper.

6.2.2 Add 250 ml acetone:double distilled water extraction solvent (5.9).

6.2.3 Shake intensively by hand for the first 15 to 30 sec and then for 30 min with a shaker (4.1).

6.2.4 Filter the extract using a pre-folded filter paper (4.2).

6.2.5 Pipette 5 ml of the clear filtrate into a 100 ml volumetric flask and make up to the mark with PBS or double distilled water (The dilution solvent – PBS or double distilled water – must be selected according to the specifications of the immunoaffinity column's manufacturer. Unless otherwise stated the dilution must be made with PBS).

If the solution is not clear, re-filter through a glass fibre filter (4.4) and apply exactly 50 ml of the clear filtrate to a reservoir that is placed on a conditioned immunoaffinity column (If the solution is clear the diluted solution can be directly applied on the immunoaffinity column).

6.2.6 Apply the solution on the column as described in section 6.3.

6.3 *Immunoaffinity clean up*

NOTE: Methods for conditioning, loading, washing and eluting vary slightly between immunoaffinity column manufacturers and therefore the specific instructions supplied with the columns should be followed precisely. In general, procedure involves sample extraction with aqueous methanol, filtration or centrifugation, possible sample dilution with PBS or double distilled water, loading under pressure onto (possibly pre-washed) column, washing of column with double distilled water and elution of aflatoxin B1 with methanol or acetonitrile.

6.3.1 Pass the filtrate through the column by gravity at a flow rate of approximately 1 drop/second (approximately 3 ml/minute). Do not exceed a flow rate of 5 ml/minute.

6.3.2 Wash the column with approximately 20 ml of double distilled water (5.8), applied in two portions of approximately 10 ml at a flow rate of 3 ml/minute and dry by applying a light vacuum for 5–10 seconds or passing air through the immunoaffinity column by means of a syringe for 10 seconds.

6.3.3 Elute the aflatoxin B_1 in a two-step procedure:

Apply 0.50 ml methanol on the column and let it pass through by gravity. Collect the eluate in a 5 ml volumetric flask (4.7). Wait for 1 minute and apply a second portion of 1.25 ml methanol. Collect most of the applied elution solvent by passing air through, after most of the solution has passed through by gravity.

Option A (recommended)

NOTE: This option is recommended, but requires an appropriate fluorescence detector and injection system. Option B only applies if the detector signal is low for the analysis according to option A.

6.3.4 Collect the eluate in a 5 ml volumetric flask (4.7).

6.3.5 Fill the flask to the mark with double water and shake well. If the solution is clear it can be used directly for HPLC analysis. If the solution is not clear, pass it through a filter unit (0.45 µm) (4.14) prior to HPLC injection.

NOTE: The injection by total loop mode guarantees maximum accuracy. It is recommended (depending on the injection system, e.g. syringe or autosampler) to take a sample volume of 3 times the injection loop size and to inject at least 2/3 of this volume into the valve, to ensure that the middle fraction remains in the injection loop. Thus, the loop is rinsed with the injection solvent while enough solvent remains in the valve.

Option B (only if applicable)

NOTE: If the detector signal is very low to guarantee the required level for Relative Standard Deviation (RSD) an additional evaporation step may be included to meet the required RSD (10% of a multiple injection (n = 10) of a standard solution of aflatoxin B1 with a concentration equivalent to a contamination level of 1 ng/g).

6.3.6 Collect the aflatoxin containing methanol eluate from the immunoaffinity column in an acid washed glass vial.

6.3.7 Evaporate the eluent to dryness under a gentle stream of nitrogen at 40 °C.

6.3.8 Re-dissolve the aflatoxin in an aqueous methanol solution (35%). Use exactly the same volume for the evaporated sample residues as you will use for the evaporated calibration solution. The volume for re-dissolving (final volume) will depend on the size of your injection loop. Use the total loop mode for injection as described in option A.

6.4 *Post column derivatisation*

When using PBPB mount the mixing T-piece and reaction tubing mentioned under 4.11, and then operate using the following parameters:

Flow rates: 1 ml/minute (mobile phase 5.12)
0.30 ml/minute (reagent 5.14)

When using electrochemically generated bromine (KOBRA cell) follow the instructions for the installation of the cell as supplied by the manufacturer and operate using the following parameters:

Flow rate: 1 ml/min (mobile phase 4.13)

Current: 100 µA

6.5 Calibration curve

Calibration curve should be prepared using the working calibration solutions described (5.17). These solutions cover the range of 0.5–5.5 µg/kg for aflatoxin B1. Make the calibration curve prior to analysis according to the table (5.17) and check the plot for linearity. Linear regression should be performed using a scientific calculator or statistical program.

NOTE: In case the content of aflatoxin B_1 in the sample is outside of the calibration range, an appropriate calibration curve must be prepared. Alternatively the injection solution for HPLC analysis can be diluted to an aflatoxin B_1 content appropriate for the established calibration curve.

6.6 Spiking procedures for recovery determination

To determine the recovery, spike aflatoxin standard solution to initial weight of an aflatoxin-free material. The spiking level should be within the calibration range (preferably mid-range). Take care that not more than 2 ml of the spiking solvent is added (solution must have an adequate concentration of aflatoxin B_1) and that the subsequent evaporation takes place in the dark and should last 0.5–2 hours.

7. Calculation

Plot the signal as x-axis (height or area) against the concentration of aflatoxin B_1 [ng/ml] as y-axis from the calibration solution (section 6.5). Draw the calibration curve and calculate slope (*a*) and intercept (*b*) using linear regression.

$$y = ax + b$$

Using the resulting function calculate the concentration of aflatoxin B_1 in the measured solution.

Calculation of the aflatoxin B_1 concentration of the injected solutions from the calibration curve (function) obtained by linear regression:

$$\rho_{smp} = a \cdot A_{smp} + b$$

$$\omega_{conta} = \frac{\rho_{smp} \cdot V_S \cdot V_E \cdot V_D}{m \cdot V_{AE} \cdot V_{A/AC}}$$

$$\omega_{conta} = \frac{\rho_{smp} \cdot 100 \cdot V_E}{m}$$

where,

m = sample material taken for analysis [g] - (50 g),
V_S = solvent taken for extraction [ml] - (250 ml),
V_{AE} = aliquot taken form extract [ml] - (5 ml),
V_D = volume achieved after dilution with PBS (water) [ml] - (100 ml),
$V_{A/AC}$ = aliquot taken for the immunoaffinity clean-up [ml] - (50 ml),
V_E = final volume achieved after elution from immunoaffinity column [ml],
ρ_{smp} = concentration of aflatoxin calculated from linear regression [ng/ml],
ω_{conta} = contamination of sample material with aflatoxin B_1 [µg/kg], and
A_{smp} = area or height of aflatoxin peak obtained from the measured solution [units].

Bear in mind, that for sample and standard solutions the same volume must be injected to comply with the formula.

8. Quality control

8.1 Accuracy

Recovery of the spiked sample (6.6) must account for 60 to 120%, otherwise the series has to be repeated. This working control should be run with each set and compared to limits on established control chart.

8.2 Precision

Inject a mid-ranged calibration standard as a check after roughly every 5 samples to

evaluate the change in peak area. The area should not change more than 10% from the standard peak area in the calibration curve. Check this also for AFB_1, AFB_2, AFG_1 and AFG_2 if they are also being determined.

9. Remarks

9.1 Concerning the loading capacity of immuno affinity columns refer to manufacturer's specifications.

9.2 Before applying the HPLC-confirmation it is possible to screen for aflatoxins with different test-kits. Several ELISA-tests are commercially available.

9.3 The test report shall contain the following data:

- information necessary for the identification of the sample (kind of sample, origin of sample, designation);
- a reference to this method;
- the date and type of sampling procedure (if known);
- the date of receipt;
- the date of test;
- the test results and the units in which they have been expressed;
- recovery rate of the working control sample (8);
- a statement whether the results are corrected for recovery; and
- particular points observed in the course of the test; operations not specified in the method or regarded as optional, which might have affected the results.

10. References

AOAC 2005.08. 2008. *Aflatoxins in corn, raw peanuts and peanut butter using liquid chromatography with post-column photochemical derivatization, Section number 49.2.18A*. Gaithersburg, MD, USA

AOCS. 2005. *Analysis of aflatoxin using HPLC post-column photochemical derivatization, recommended practice Aa 11-05*. Champaign, USA

EN 17375. 2006. *Animal feeding stuffs – Determination of aflatoxin B_1, International Organization for Standardization*. Geneva, Switzerland.

FUMONISINS – HPLC METHOD

1. Principle

Fumonisins B_1 and B_2 (FB_1, FB_2) are extracted from the feed sample with aqueous methanol. The extract is purified using immunoaffinity columns. The analytes are separated by reverse-phase high-performance liquid chromatography (RP-HPLC) and detected by their fluorescence after either pre- or post column derivatisation with o-phtalaldehyde (OPA).

2. Scope

This procedure is most appropriate for the analysis of fumonisins in whole corns and compound animal feeds. Limit of quantification should be at least 3 mg/kg for the sum of fumonisins, but depending on the equipment it is possible to achieve 0.5 mg/kg and lower quantification limits for each fumonisin.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Mill.
4.2 Tumble Mixer.
4.3 Vortex mixer.
4.4 Laboratory shaker.
4.5 250 ml flasks with screw caps.
4.6 Graduated cylinders of 5, 50, 1000 and 2000 ml capacity.
4.7 Graduated pipettes (Class A) of 2, 10 and 50 ml capacity.
4.8 Analytical balance, accurate to 0.1 mg.
4.9 Glass microfibre filter, binder-free with a pore size of *ca* 2 µm.
4.10 Filter funnel of appropriate size.
4.11 Auto sampler vials of appropriate size with caps.
4.12 Reservoirs of appropriate size for immunoaffinity columns, with adapter for connecting to top of immunoaffinity columns.
4.13 Volumetric flasks (Class A) 2, 5, 10 and 20 capacity.
4.14 Gastight glass syringes and/or positive displacement pipettes capable of precisely dispensing the volumes: 5, 50, 125, 160 and 500 µl and 1 ml.
4.15 Support stand of appropriate size for immunoaffinity columns.
4.16 HPLC instrumentation, comprising the following:
- 4.16.1 Solvent delivery system capable of generating a binary gradient with sufficient precision at the required pressures, e.g. Agilent Series 1200 pump.
- 4.16.2 Auto sampler capable of injecting sufficient volumes of injection solution with sufficient repeatability and, for pre-column derivatisation capable of mixing reagent and sample solution before injection, e.g. Agilent Series 1200 ALS.
- 4.16.3 Chromatographic column: Any column which provides symmetric peak (peak asymmetry factor $0.9 < As < 1.4$ at 10% of full height), sufficient retention ($k > 2$), and resolution ($Rs > 1$) for FB_1 and FB_2, e.g. Agilent Zorbax SB-C18 4.6 x 150 mm, 3.5 µm.
- 4.16.4 Fluorescence detector: capable of providing the required excitation and emission wavelengths and equipped with a flow cell of appropriate size, e.g. Agilent Series 1200 FLD or Waters 474.
- 4.16.5 Post-column derivatisation system (not necessary if pre-column derivatisation is used): either a commercial unit or self-assembled; if self-assembled the following items are needed:
  - Reagent pump capable of delivering a constant pulsation-free flow of the derivatisation reagent against the required pressures;
  - PEEK tubing of the outer diameter required by the HPLC system in use and varying inner diameters, e.g. 1/16" OD (outer diameter), 0.04", 0.02" ID, 0.01" ID or 0.005" ID; and

- Mixing Tee: small internal volume PEEK (polyetheretherketon), e.g. VICI JR-9000-0665.

4.17 Nylon filter 0.45 µm.

5. Reagents

All solutions are made with HPLC grade solvents and reagent grade materials unless otherwise noted. Only double distilled water or water of at least grade 2 as defined in EN ISO 3696 shall be used.

5.1 Double distilled or deionised water.
5.2 Methanol.
5.3 Acetonitrile.
5.4 Potassium chloride (KCl).
5.5 Sodium chloride (NaCl).
5.6 Disodium hydrogenphosphate dodecahydrate ($Na_2HPO_4.12\ H_2O$).
5.7 Disodium tetraborate decahydrate ($Na_2B_4O_7.10\ H_2O$).
5.8 Sodium carbonate (Na_2CO_3).
5.9 Boric acid (H_3BO_3).
5.10 Potassium sulphate (K_2SO_4).
5.11 N-Acetyl-L-Cystein (NAC).
5.12 o-Phtalaldehyde (OPA).
5.13 β-Mercaptoethanol (BME).
5.14 Formic Acid (98–100%).
5.15 Phosphate buffered saline (PBS) concentrate
Dissolve the following in 1800 ml of double distilled water (5.1):
4 g KCl (5.4)
160 g NaCl (5.5)
72 g $Na_2HPO_4.12\ H_2O$ (5.6)
Adjust to pH 7.4 with 10 M HCl and make up the volume to 2000 ml with double distilled water.
5.16 PBS Ready to use: Dilute 100 ml of PBS concentrate (5.15) to 1000 ml with double distilled water (5.1) or PBS tablet e.g. Sigma P4417 (one tablet dissolved in 200 ml of double distilled water (5.1) yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25 °C).
5.17 Diluent: Mix methanol (5.2) and double distilled water (5.1) in 1:1 (v/v).
5.18 Extraction solvent:
Mix methanol (5.2) and PBS (5.16) in 1:1 (v/v).
5.19 Reaction buffers for post- and pre-column derivatisation
5.19.1 Post-column derivatisation: 0.006 M OPA, 0.006 M NAC, 0.384 M sodium carbonate, 0.216 M boric acid and 0.108 M potassium sulphate
- Dissolve 40.7 g sodium carbonate (5.8), 13.4 g boric acid (5.9) and 18.8 g potassium sulphate (5.10) in 1 litre of double distilled water (5.1);
- Stir for 10 minutes;
- Add 800 mg of OPA (5.12) in 1 litre of the above solution;
- Add 1 g of NAC (5.11) in 1 litre of the above solution;

- Stir for 10 minutes;
- Place in Ultrasonic water bath for 15 minutes.
- Stir for 10 minutes; and
- Place in Ultrasonic water bath for 15 minutes. Filter the solution through a 0.45 µm nylon filter (4.17).

NOTE:

- Proper dissolution of the OPA is very important
- The reaction buffer should not be changed within a sequence of HPLC runs
- Prepare fresh for every sequence of HPLC runs

5.19.2 Pre-column derivatisation: 0.1 M OPA, 0.24 M BME, 0.08 M disodium tetraborate, 16.7% methanol

- Dissolve 40 mg OPA (5.12) in 1 ml methanol (5.2);
- Mix until completely dissolved;
- Add 5 ml of a 0.1 M solution of disodium tetraborate decahydrate (3.8 g/100 ml; 5.7);
- Mix thoroughly;
- Add 50 µl of BME (5.13); and
- Mix thoroughly.

Alternatively, phthaldialdehyde reagent (Sigma-Aldrich P0532) can be used

5.20 FB_1 and FB_2 stock solution: A certified solution of Fumonisin FB_1 and FB_2 of *ca* 50 µg/ml each in an appropriate solvent. Take exact concentration from the certificate.

5.21 FB_1 and FB_2 diluted stock solution for calibration: Add 160 µl of the FB_1 and FB_2 stock solution (5.20) into a 2 ml volumetric flask (4.13). Make up to 2 ml with the diluent (5.17). This will result in 2.0 ml of a 12.5 fold dilution of solution 5.20.

NOTE: The above solutions (5.20, 5.21) may also be prepared gravimetrically by accurately weighing the dry substance and the solvent used to dissolve it.

5.22 Calibration solutions: From the diluted stock solutions (5.21) prepare 5 levels of calibration solutions by adding the volumes of the diluted stock solution listed in Table 4 into a volumetric flask (4.13) of the indicated volumes and make up to the mark with the diluent (5.17).

Calculate the concentrations of FB_1 and FB_2 for the different calibration levels by dividing the certified or calculated concentrations of the stock solution (5.20) by the final dilution stated in Table 4. Should you observe saturation of the detector signal at the highest calibration level, dilute 250 µl of the diluted stock solution (5.21) into 2 ml, for a final dilution of 100.

These calibration levels are recommendations and may be adjusted to individual needs.

5.23 Immunoaffinity columns. The immunoaffinity columns must contain a stationary phase with immobilised monoclonal antibodies specific to, at least, FB_1 and FB_2.

To be suitable for this method they must meet the requirements stated below:

An aliquot of an extract of a fumonisin-free representative compound animal feed material is spiked with FB_1 and FB_2 at either 920 (high) or 110 (low) ng/ml for the sum of the both. Then dilute 5 ml of this spiked extract to a total volume of 50 ml (see 6.2).

TABLE 4
Fumonisin standard concentrations

Calibrant No.	Diluted stock solution (5.21) [µl]	Volumetric flask (4.13) [ml]	Final dilution of stock solution (5.20)
1	50	20.0	5000
2	125	10.0	1000
3	125	5.0	500
4	500	2.0	50
5	1000	2.0	25

Following the procedures described in 6.3 and 6.4 this will result in expected concentrations in the injection solutions of either 460 or 55 ng/ml for the sum of FB_1 and FB_2.

After measuring these solutions the observed concentrations of FB_1 and FB_2 can be calculated using equations 1 and 2 (given in section 7). Dividing the sum of the observed concentrations of FB_1 and FB_2 by the expected concentrations will result in the yield from the immunoaffinity columns. These yields must be 99 ± 18% (U, k = 2) at the high level and 118 ± 18% (U, k = 2) at the low level.

The above column test should be performed for each level on at least three randomly selected columns of every new batch of immunoaffinity columns which will be used. Should the tested batch not meet the above requirements either a new batch should be obtained or the conditions described in 6.3 need to be adjusted so that the requirements are met (the user instructions supplied with the columns are a good starting point).

Any change in the clean-up procedures will necessitate a revalidation of the clean-up and all subsequent steps (chromatography).

6. Procedure

6.1 Extraction of FB_1 and FB_2

6.1.1 Weigh 20 g of the test sample into a large enough container with lid, e.g. 250 ml flask (5.5).

6.1.2 Add 200 ml of extraction solvent (4.18), cap the flask and shake vigorously by hand, so that the material disperses evenly.

6.1.3 Put it on a shaker (5.4) for 120 minutes. Choose speed such that the material is mixed well without collecting in the top of the flask.

6.1.4 Allow the extracted sample to settle after shaking.

6.1.5 Take 5 ml of the extract (6.1.4) and dilute with PBS (4.16) to a total volume of 50 ml and mix.

6.1.6 Prepare a filter funnel (5.10) with a glass microfibre filter (5.9).

6.1.7 Filter the diluted supernatant of the extracted sample into a new flask (5.5).

6.1.8 The diluted filtered extract may be stored at 4–10 °C overnight.

6.1.9 In case of a highly contaminated material above 10000 µg/kg (see 8) take 10 ml of the stored filtered diluted extract and dilute again with PBS (4.16) to a total volume of 50 ml and mix.

6.2 Clean up

6.2.1 Take one immunoaffinity column (IAC, 4.23) per extract.

6.2.2 Attach a reservoir (5.12), do not empty storage solution from column.

6.2.3 To the reservoir add 25 ml of the filtered diluted extract (6.1).

6.2.4 Open the column outlet.

6.2.5 Allow everything to pass slowly through the column. Flow rate should be one to two drops per second.

6.2.6 After the extract has passed completely through the column, wash the immunoaffinity column with 10 ml of PBS (4.16).

6.2.7 Pass air through the immunoaffinity column (e.g. using a properly fitted large syringe) in order to expel excess PBS.

6.2.8 Place a 5 ml volumetric flask (5.13) or a 5 ml graduated cylinder (5.6) underneath the immunoaffinity column and add 5 x 500 µl of methanol (4.2) to the immunoaffinity column (add next aliquot only after the previous has completely passed through the column).

6.2.9 Collect all the eluent in the volumetric flask (5.13) or graduated cylinder (5.6).

6.2.10 Add 2 ml of double distilled water (4.1) to the immunoaffinity column after all of the methanol (4.2) has passed through the column.

6.2.11 Continue to collect the eluent in the same volumetric flask or graduated cylinder.

6.2.12 Carefully pass air through the column in order to collect most of the applied water (4.1).

6.3 Test solution

6.3.1 For pre-column derivatisation: Make up the content of the volumetric flask or graduated cylinder to the 5 ml mark with double distilled water (4.1).

6.3.2 For post-column derivatisation: add 5 µl of formic acid (4.14) and make up the content of the volumetric flask or graduated cylinder to the 5 ml mark with double water (4.1).

6.3.3 Mix the content of the volumetric flask or graduated cylinder and transfer an aliquot to an autosampler vial (5.11).

6.3.4 This test solution may be stored at 4–10 °C for up to 2 days.

6.4 Spiking procedure

To determine recovery spike a fumonisin-free representative feed material with FB_1 and FB_2 stock solution (4.20) or a dilution thereof. The spiking level should be within the calibration range (preferably mid-range). The concentration of the solution used should be such that not more than 2 ml is added. Leave the spiked sample to stand for a period of 30 minutes to ensure evaporation of the solvent.

6.5 HPLC operating conditions

Operation conditions described below work well with the equipment listed in 4.16. It could be that you will have to make adjustments if you are using different equipment to obtain appropriate resolution and retention (4.16.3). These adjustments could be in the injector program, injection volume, percentage of organic modifier in isocratic or gradient mode, flow rate, and/or the column temperature.

TABLE 5
Gradient settings (HPLC) using pre-column derivatisation

Time [min]	B [%]
0	69.5
14	79
14.01	100
17.01	100
17.02	69.5
20	69.5

6.5.1 Pre-column derivatisation

Using the equipment outlined in 4.16, the following conditions have shown to produce satisfactory results.

- Auto sampler injector program:
- Aspire 20 µl pre-column reaction buffer (5.19.2);
- Aspire 40 µl test solution (6.4);
- Aspire 20 µl pre-column reaction buffer (5.19.2);
- Mix 20 times; and
- Inject all.

The above can be done manually (adjusting the total volume while maintaining the relative volumes if necessary) if it is ascertained that the solution is injected within 3 minutes after mixing. It is also important that the time period between mixing and injection is the same for all test and calibration solutions.

- Injection volume: 80 µl
- Column temperature: 40 °C
- Flow : 1.0 ml/minute
- Fluorescence detector: Excitation λ: 335 nm; Emission λ: 440 nm (check using a wavelength spectrum for the fluorescence detector in use).
- Mobile phase: A: 0.5% formic acid (5.14) in double distilled water (5.1)
  B: 0.5% formic acid (5.14) in methanol (5.2)
- Gradient settings (HPLC dwell volume 0.8 ml), see Table 5:

Instruments with different dwell volume will need adjustment of the gradient to achieve similar separation. The aim should be to achieve an apparent capacity factor (k) at elution for FB_1 of > 3.

6.5.2 Post-column derivatisation

Instructions for self-assembled system:

The flow path to the chromatographic column (4.16.3) is unchanged from normal operation. The outlet of the column is connected to one of the outside ports of a mixing Tee (4.16.5.3). The tubing from column to mixing Tee should be as short as possible.

TABLE 6
Gradient settings using post-column derivatisation

Time [min]	B [%]
0	34
13	34
13.01	95
16	95
16.01	34
19	34

The other outside port of the mixing Tee is connected to the outlet of a pump (4.16.5.1) delivering the reagent flow. This connection should be made of a long piece of 0.005" ID PEEK tubing (4.16.5.2) so that a sufficient back pressure is created for the reagent pump to work properly. It is of utmost importance that the reagent flow is delivered pulsation-free. A slight pulsation can be minimised by introducing a large damping volume between the pump and the back pressure creating PEEK tubing. Large ID PEEK tubing can serve this purpose.

The remaining centre port of the mixing Tee is connected through a reagent loop to the fluorescence detector.

The length, and therefore the volume, of this reagent loop is a balance between retaining the resolution of the chromatographic column (short) and achieving complete reaction (long). The internal diameter is of lesser importance. If chosen too small excessive back pressure will be created. Satisfying results were achieved with a 2.5 m length of 0.02" ID PEEK tubing.

Using the equipment outlined in 4.16, the following conditions have shown to produce satisfactory results.

- Injection volume: 50 µl
- Column temperature: 45 °C
- Flow: 1.2 ml/minute (mobile phase); 0.45 ml/minute (post-column reagent; 5.19.1).
- Fluorescence detector: Excitation λ: 335 nm; Emission λ: 440 nm (check using a wavelength spectrum for the fluorescence detector in use).
- Mobile phase: A: 0.1% formic acid (5.14) in double distilled water (5.1)
  B: 0.1% formic acid (5.14) in acetonitrile (5.3)

This separation is isocratic but to avoid accumulation of matrix components a step of 95% B is included. The percentage of organic modifier should be adjusted such that the capacity factor (k) for FB_1 is > 2.

6.6 Determination of fumonisins in test solutions

Inject aliquots of the test solutions (6.4) into the HPLC using the same conditions as used for the calibration solutions (5.22).

6.7 Batch (Sequence) composition

Always start a batch of measurements with a reagent blank to check the system. Subsequently inject the calibration solutions. Before the injection of the first test solution the reagent blank should be injected to prove that there is no carry-over of analytes. The test solutions should be run in duplicate and reruns of the calibration solutions should be interspersed at regular intervals. The frequency of these calibration reruns depend on the stability of your chromatographic system.

6.8 Calibration

Plot the signals (peak area or height) of all the measured calibration solutions against the corresponding concentrations for FB_1 and, separately, for FB_2. Do not use means of the multiple injections. With linear regression, estimate slope and intercept of each of the two calibration functions (FB_1 and FB_2). Check for significance of the intercept and for linearity (use e.g. a residuals vs. fitted-values plot).

6.9 Peak identification

Identify Fumonisin B_1 and B_2 peaks in the test solution by comparing the retention times with those of the closest calibration solution in the batch. The signal (peak area or height) of FB_1 and FB_2 in the test solution must fall within the calibration range. If the FB_1 and/or FB_2 signal in the test solution exceeds the signals of the highest calibration solution, the test solution should be diluted with diluent (5.17) to bring it within the calibration range, and be re-analysed. The dilution factor must be incorporated into all subsequent calculations.

7. Calculation

Using the estimated slopes and intercepts (if significant, otherwise use zero) from linear regression (6.9) calculate the concentrations of FB_1 (c_{FB1}) and FB_2 (c_{FB2}) in the test solutions (6.4) from the mean signal of the duplicate injections as follows:

$$c_{FB1} = \frac{\overline{signal}_{FB1} - \mathrm{int}\,ercept_{FB1}}{slope_{FB1}} [ng/ml] \quad (1)$$

$$c_{FB2} = \frac{\overline{signal}_{FB2} - \mathrm{int}\,ercept_{FB2}}{slope_{FB2}} [ng/ml] \quad (2)$$

If the test solution was diluted because of a signal above the calibration range (6.10) multiply c_{FB1} and c_{FB2} with the dilution factor.

To calculate the mass fractions (w_{SMP}) of the analytes in the original materials use the following equation:

$$w_{SMP} = \frac{c \cdot V_5 \cdot V_3 \cdot V_1}{V_4 \cdot V_2 \cdot m_{SMP}} [ng/g \text{ or } \mu g/kg] \quad (3)$$

where,

c = calculated concentration of FB_1 (1) or FB_2 (2), possibly corrected for dilution c_{FB1} and c_{FB2},
m_{SMP} = weight of the test material used for extraction (20 g),
V_1 = total volume of the extraction solvent (200 ml),
V_2 = volume of the aliquot of the filtered raw extract used for dilution (5 ml),
V_3 = total volume of the diluted filtered raw extract (50 ml),
V_4 = volume of the aliquot of the diluted filtered raw extract applied to IAC (25 ml), and
V_5 = total volume of the test solution (5 ml).

If weight of the test material and the volumes described here are kept the same, the above equation (3) can be simplified to:

$$w_{SMP} = c \cdot 20 \; [\mu g/kg] \tag{4}$$

Should the result of equation 4 be larger than 10000 µg/kg or if it is known before that the contamination level might exceed that value clean-up the respective diluted filtered extract (6.1) using an additional dilution (additional dilution factor 50/10 = 5). The simplified equation will then be:

$$w_{SMP} = c \cdot 20 \cdot 5 = c \cdot 100 \; [\mu g/kg] \tag{5}$$

Carry out the above calculations for FB_1 and FB_2. Sum of both will then be calculated as follows:

$$w_{SMP} = w_{SMP,\,FB1} + w_{SMP,\,FB2} \; [\mu g/kg] \tag{6}$$

8. Quality control

8.1 Accuracy
Recovery of the spiked sample (6.4) must account for 60–120%, otherwise the series has to be repeated. This working control should be run with each set and compared to limits on established control chart.

8.2 Precision
Inject a mid-ranged calibration standard as a check after roughly every 5 samples to evaluate the change in peak area. The area should not change more than 10% from the standard peak area in the calibration curve. Do this for all fumonisins, FB_1 and FB_2.

9. Remarks

9.1 Applying this method it is also possible to determine fumonisin B_3 (FB_3).

9.2 Concerning the loading capacity of immunoaffinity columns refer to manufacturer's specifications.

9.3 Before applying the HPLC procedure, samples can be screened for fumonisins with different test-kits. For example several ELISA-tests are commercially available (see link list in the aflatoxin method). Refer to the manufacturer's product and procedure descriptions.

9.4 The test report shall contain the following data:

- information necessary for identification of the sample (kind of sample, origin of sample, designation);
- a reference to this method;
- the date and type of sampling procedure (if known);
- the date of receipt;
- the date of test;
- the test results and the units in which they have been expressed;
- recovery rate of the working control sample (8);
- a statement whether the results are corrected for recovery;
- particular points observed during the course of the test; operations not specified in the method or regarded as optional, which might have affected the results.

10. References

EN 16006. 2011. *Animal feeding stuffs – Determination of the sum of Fumonisin B1 and B2 in compound animal feed with immunoaffinity clean-up and RP-HPLC with fluorescence detection after pre- or postcolumn derivatisation*. Geneva, Switzerland.

Commission Regulation (EC). 2006. No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Union*, 2006. 49 (L70), pp. 12–34.

AOAC. 2000. Natural poisons. Fumonisin B1, B2 and B3 in corn. *Official Methods of Analysis. Method 995.15*. Association of Official Analytical Chemists, Inc., Gaithersburg, MD, USA.

ZEARALENONE (ZON) – HPLC METHOD

1. Principle

Zearalenone is extracted from the sample using aqueous methanol. The extract is diluted with phosphate buffered saline and zearalenone is isolated on an immunoaffinity column containing antibodies specific for zearalenone. The analyte is quantitatively determined by high performance liquid chromatography (HPLC) with fluorescence detection (FLD).

2. Scope

This Standard is applicable to the determination of zearalenone in animal feed at concentrations from 30 to 3000 µg/kg.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

Usual laboratory equipment and in particular the following.

4.1 Analytical balance, accurate to 1.0 mg.
4.2 Horizontal or vertical shaker.
4.3 Homogeniser/high speed blender.
4.4 Vortex mixer, or equivalent.
4.5 pH meter.
4.6 Mill (various screens).
4.7 Tumble mixer.
4.8 Glass vials, various sizes.
4.9 Graduated pipettes of 5 ml and 50 ml capacity.
4.10 Graduated cylinders with and without stoppers of 5 ml and 250 ml capacity.
4.11 Volumetric flasks of 3 ml, 5 ml and 10 ml capacity.
4.12 Beaker of 250 ml capacity.
4.13 Conical or screw cap flasks of 100 ml and 250 ml to 500 ml capacity.
4.14 Glass funnels of appropriate size.
4.15 Folded filters, cellulose (*ca* 30 µm pore size) for the glass funnels (4.14).
4.16 Filter disks, binder-free glass microfibre (< 2 µm pore size) of appropriate size for the solvent vacuum filtration system (4.22).
4.17 Pipettors or gas-tight glass syringes of 100 µl and 500 µl and 1 ml.
4.18 Vacuum manifold or Automated SPE Vacuum System, capable of accommodating the immunoaffinity columns.
4.19 Reservoirs, of appropriate volume with attachments to fit the immunoaffinity columns.
4.20 Plastic syringes, 5 ml.
4.21 Vacuum pump capable of generating sufficient vacuum for the solvent vacuum filtration system (4.22).
4.22 Solvent vacuum filtration system fitted with glass microfibre filter (4.16).
4.23 HPLC syringe filter unit, polyamide (nylon) with 0.45 µm pore size.
4.24 Ultrasonic bath.
4.25 HPLC apparatus, comprising of the following:
- 4.25.1 Injection system, manual or autosampler, with loop suitable for 100 to 300 µl injections.
- 4.25.2 Pump, isocratic, pulsation-free, capable of maintaining a volume flow rate of 0.5 to 1.5 ml/minute.
- 4.25.3 Analytical reversed phase HPLC column, generally all RP-columns are suitable that allow a sufficient separation of zearalenone from other interfering components, for example Phenomenex Octadecylsilane (ODS)3-Prodigy (150 mm x 4.6 mm ID.), 5 µm particle size, 250 Å pore size, or Spherisorb Octadecylsilane (ODS)2-Excel (250 mm x 4.6 mm ID), 5 µm particle size, 250 Å pore size have been found to be suitable.
- 4.25.4 Pre-column (optional), appropriate for the analytical column used.
- 4.25.5 Fluorescence detector, fitted with a flow cell and suitable for measurements with excitation wavelength of 274 nm and emission of 446 nm.
- 4.25.6 Data system, integrator or PC workstation.

4.26 UV spectrophotometer for checking the concentration of the stock solution (5.15).

5. Reagents

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and only double distilled water or water of grade 1 as defined in EN ISO 3696 (1995). Solvents shall be of HPLC grade.

5.1 Acetonitrile.

5.2 Methanol, technical grade.

5.3 Methanol, HPLC grade.

5.4 Sodium chloride.

5.5 Disodium hydrogen orthophosphate.

5.6 Potassium dihydrogen phosphate.

5.7 Potassium chloride.

5.8 Hydrochloric acid (32%).

5.9 Phosphate buffered saline (PBS).

Dissolve 8 g sodium chloride (5.4), 1.2 g disodium hydrogen orthophosphate (5.5), 0.2 g potassium dihydrogen phosphate (5.6) and 0.2 g potassium chloride (5.7) in 1 litre of double distilled water. Adjust the pH to 7.4 with hydrochloric acid (5.8).

NOTE: Commercially available phosphate buffered saline tablets with equivalent properties may be used.

5.10 Extraction solvent, methanol:double distilled water (75:25, v/v)

Mix 75 parts by volume methanol (5.2) with 25 parts by volume of double distilled water.

5.11 Washing solvent, methanol:PBS (15:85, v/v)

Mix 15 parts by volume methanol (5.3) with 85 parts by volume PBS (5.9).

5.12 Injection solvent for HPLC analysis, methanol:double distilled water (50:50, v/v)

Mix 50 parts by volume methanol (5.3) with 50 parts by volume double distilled water.

5.13 HPLC mobile phase, methanol:double distilled water (75:25, v/v)

Mix 75 parts by volume methanol (5.3) and 25 parts by volume double distilled water. Mix well and degas.

5.14 Zearalenone, minimum purity of 98%.

5.15 Zearalenone (ZON) stock solution, 10 µg/ml

Add 4.0 ml of acetonitrile (5.1) to 5 mg of zearalenone (5.14) for a standard solution of 1.25 mg/ml. Dilute 800 µl of the 1.25 mg/ml standard solution to 5.0 ml with acetonitrile (5.1) for a standard solution of 200 µg/ml. Dilute 250 µl of the 200 µg/ml standard solution to 5.0 ml of acetonitrile (5.1) to create the stock solution of 10 µg/ml.

To determine the exact concentration record the absorption curve of this 10 µg/ml stock solution using a spectrophotometer (4.26) in the range of 200 nm to 300 nm in a 1 cm quartz cell with acetonitrile (5.1) as reference. Determine the absorption of the second maximum at l = 274 nm. Calculate the mass concentration of zearalenone, ρ_{ZON}, in micrograms per ml using the following equation:

$$\rho_{ZON} = \frac{A_{\max} \cdot M \cdot 100}{\kappa \cdot d}$$

where,

A_{max} = absorption determined at the second maximum of the absorption curve (274 nm);

M = molar mass of zearalenone (M = 318.4 g/mol);

κ = molar absorption coefficient of zearalenone (1262 m^2/mol);

d = optical path length of the quartz cell in centimetres (1 cm)

Store standard solutions at below –18 °C.

5.16 ZON spiking solution

The calibrated stock solution (see 5.15). This solution is stable for 2 months if stored at below –18 °C.

5.17 ZON working solution

Transfer an aliquot of the calibrated stock solution (5.15), equivalent to 10 µg of ZON, into a volumetric flask (4.11). Add acetonitrile (5.1) to make the total volume up to 5 ml. This is a 2 µg/ml working solution. This solution is stable for 2 months if stored at below –18 °C.

5.18 ZON Calibration solutions for HPLC

Prepare 5 HPLC calibration solutions in separate 10 ml volumetric flasks (4.11) by pipetting the volumes shown in Table 7. Make each standard up to 10 ml with HPLC injection solvent HPLC (5.12).

5.19 ZON immunoaffinity clean-up columns

The immunoaffinity column contains antibodies raised against zearalenone. The column should have a capacity of not less than 1500 ng of zearalenone and a recovery of not less than 70% when 75 ng of zearalenone are applied in 10 ml of washing solvent (5.11).

6. Procedure

6.1 Sample Preparation

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Samples should be finely ground and thoroughly mixed using a mill (4.6) and a tumble mixer (4.7) or another process that has been demonstrated to give complete homogenisation before a test portion is removed for analysis. In all instances if the sample has been frozen, allow it to thaw completely before sampling. Stir the sample thoroughly before removing an analytical test portion.

TABLE 7
ZON Calibration concentrations

Calibration solution	Volume of ZON working solution (5.17) [µl]	ZON concentration [ng/ml]
1	50	10
2	250	50
3	450	90
4	650	130
5	850	170

6.2 Extraction

6.2.1 Weigh 20 g (recorded to 2 decimal places) test portion into a screw cap flask of 250 ml to 500 ml (4.13).

6.2.2 Add 150 ml the extraction solvent (5.10).

6.2.3 Mix briefly by hand to obtain a homogeneous suspension, then either shake for 1 hour on a shaker (4.2), or place in an Ultrasonic water bath for 15 minutes (4.24).

6.2.4 Shake on a shaker (4.2) for another 15 minutes.

6.2.5 Filter extract through folded filter paper (4.15) and collect the extract in a screw cap flask of 100 ml (4.13).

6.2.6 Transfer exactly 30 ml (or 3 ml in case of results above 500 µg/kg) of the filtered extract into a 250 ml graduated cylinder with stopper (4.10). Dilute the extract in the cylinder with PBS (5.9) to the 150 ml mark.

6.2.7 Mix and filter approximately 20 ml of this diluted extract through a glass microfiber filter (4.16) into a glass beaker by applying a slight vacuum (4.22). Do not apply too strong a vacuum in the beginning of the filtration process, as this can lead to filtered extracts that are turbid after filtration.

6.2.8 Discard the first 20 ml and filter another approximately 70 ml for analysis.

NOTE: Proceed immediately with the immunoaffinity column clean-up procedure (6.3).

6.3 Immunoaffinity Column Clean-up

6.3.1 Connect the immunoaffinity column (5.19) to the vacuum manifold (4.18) and attach a reservoir (4.19) to the top of the immunoaffinity column.

6.3.2 Precondition the immunoaffinity column (5.19) with 20 ml of PBS (5.9) using a flow rate of 3–5 ml/minute.

6.3.3 Pipette 50 ml of the filtered and diluted sample extract (6.2) into the reservoir.

6.3.4 Let the extract pass through the column by gravity at a steady flow rate until all the extract has passed and the last solvent portion reaches the frit of the column. The flow rate should be 1–2 drops/second.

6.3.5 After the extract has passed through the column, wash the column with 5 ml of washing solvent (5.11) followed by 15 ml of double distilled water at a flow rate of 1–2 drops/second.

6.3.6 Remove the residual water from the column by passing 3 ml of air or nitrogen through the column (1–2 seconds). Discard all the eluent from this stage of the clean-up procedure.

6.4 Preparation of the test solution for HPLC analysis

6.4.1 Place a 5 ml graduated cylinder (4.10) or a 3 ml volumetric flask (4.11) under the column and pass 0.75 ml of methanol (5.3) through the column, collecting the eluent.

6.4.2 After the last drops of methanol have passed through the column allow the methanol to remain on the column for approximately 1 minute.

6.4.3 Add another 0.75 ml of methanol (5.3) and continue to collect the eluent.

6.4.4 Carefully pass air through the column in order to collect any residual methanol.

6.4.5 Fill the graduated cylinder/volumetric flask to the 3 ml mark with double distilled water and mix. After mixing check volume again and adjust if necessary.

6.4.6 In case of turbid samples filter the test solution through a HPLC syringe filter unit (4.23) with a plastic syringe (4.20) before injection.

NOTE: Alternatively the manual procedures described for immunoaffinity clean-up (6.3) and elution (6.4) may be performed with an automatic sample preparation unit, provided that volumes and flow rates remain unchanged.

6.5 Calibration

Prepare a calibration graph at the beginning of every day of analysis using the ZON calibration solutions (5.18). Establish the calibration curve prior to the analysis of test samples by plotting the concentration of ZON [ng/ml] (x-axis) against the peak signal as area or height (y-axis), determine slope and possible intercept with linear regression, and check for linearity using appropriate diagnostics.

6.6 Determination and identification of zearalenone in the test solution

6.6.1 Inject aliquots of the test solutions (6.4) into the HPLC using the same conditions used for the preparation of the calibration graph.

6.6.2 Identify the zearalenone peak in the test solution by comparing the retention time with that of the nearest HPLC calibration solution (5.18) injected during the HPLC analyses batch.

6.6.3 The concentration of zearalenone in the test solution must fall within the calibration range. If the zearalenone level in the solution exceeds the concentration of the highest calibration solution, the test solution should be diluted with HPLC diluent to bring it within calibration range and re-analysed. The dilution factor must be incorporated into all subsequent calculations.

6.7 HPLC operating conditions

When the column specified in 5.25.3 and the mobile phase specified in 5.13 were used, the following settings were found to be appropriate:

- Flow rate, mobile phase (column): 0.7 to 1.0 ml/minute
- Fluorescence detection, emission wavelength: 446 to 450 nm
- Fluorescence detection, excitation wavelength: 274 to 275 nm
- Injection volume: 100 to 300 µl

7. Calculation

Determine from the calibration graph the mass concentration in ng/ml of the zearalenone in the aliquot of test solution injected onto the HPLC column. Calculate the mass fraction of zearalenone, ω_{ZON}, in ng/g or µg/kg to one decimal place using the equation below:

$$\omega_{ZON} = c_{ZON} \cdot \frac{V_5}{V_4} \cdot \frac{V_3}{V_2} \cdot \frac{V_1}{m_s}$$

where,

c_{ZON} = mass concentration of ZON as determined from calibration (6.5);

V_5 = volume of the test solution (3.0 ml; 6.4.5);

V_4 = volume of the aliquot of diluted extract applied to the immunoaffinity column (50 ml; 6.3.3);

V_3 = total volume of the diluted filtered extract (150 ml; 6.2.6);
V_2 = volume of the aliquot of extract used for dilution (30 ml or 3 ml; 6.2.6);
V_1 = total volume of the extraction solvent (150 ml; 6.2.2); and
m_s = mass of the extracted sample (20.00 g, 6.2.1).

The above equation can be simplified if the described masses and volumes have been used:

$$\omega_{ZON} = c_{ZON} \cdot 2.25$$ (30 ml of extracts were diluted)

Should the above calculation render a value above 500 µg/kg then a new dilution of 3 ml of the sample extract should be prepared (see section 6.2). The simplified equation then is as follows:

$$\omega_{ZON} = c_{ZON} \cdot 22.5$$ (3 ml of extracts were diluted)

8. Quality Control

8.1 Accuracy

To determine the recovery, spike a zearalenone-free representative material with the ZON spiking solution (5.16). The spiking level should be within the calibration range (preferably mid-range). The concentration of the solution used should be such that not more than 2 ml is added. Leave the spiked sample to stand for a minimum of 30 minutes to ensure evaporation of the solvent. Recovery must account for 60 to 120% otherwise the series has to be repeated. This working control should be run with each set and compared to limits on established control chart.

8.2 Precision

Inject a mid-range calibration standard as a check after roughly every five samples to evaluate the change in peak area. The area should not change more than 10% from the standard peak area in the calibration curve.

9. Remarks

9.1 Concerning the loading capacity of immunoaffinity columns refer to manufacturer's specifications.

9.2 Before applying the HPLC procedure, samples could be screened for ZON with different test-kits. For example several ELISA-tests are commercially available (see link list in the aflatoxin method). Refer to the manufacturer's product and procedure descriptions.

9.3 The test report shall contain the following data:

- information necessary for identification of the sample (kind of sample, origin of sample, designation);
- a reference to this method;
- the date and type of sampling procedure (if known);
- the date of receipt;
- the date of test;
- the test results and the units in which they have been expressed;

- recovery rate of the working control sample (8);
- a statement whether the results are corrected for recovery;
- particular points observed during the course of the test; and
- operations not specified in the method or regarded as optional, which might have affected the results.

10. References

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DEOXYNIVALENOL (DON) – HPLC METHOD

1. Principle

Deoxynivalenol (DON) is extracted from the commodity using double distilled water. The aqueous extract is cleaned up with an immunoaffinity column to remove impurities from the sample. Subsequently DON is quantitatively determined by HPLC with UV detection.

2. Scope

This method is applicable for determination of DON in animal compound feed at concentrations from 150 µg/kg to at least 4000 µg/kg.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

Usual laboratory equipment and in particular the following:

4.1 Analytical balance, accurate to 0.001 g for sample weighing, accurate to 0.01 mg for gravimetrical preparation of the DON stock solution.
4.2 Homogeniser/high speed blender.
4.3 Laboratory shaker.
4.4 Vortex mixer, or equivalent.
4.5 Mill (various screens).
4.6 Tumble mixer.

4.7 Screw cap flasks of 250 ml and 500 ml capacity.
4.8 Funnels of appropriate size.
4.9 Filter, cellulose with *ca* 30 µm pore size.
4.10 Filter, binder-free glass microfiber with *ca* 2 µm pore size.
4.11 Volumetric flasks of 2 ml, 5 ml and 10 ml capacity.
4.12 Graduated pipettes of 1 ml and 5 ml capacity.
4.13 Adjustable pipettes or gas-tight glass syringes of 100 µl and 1 ml capacity.
4.14 HPLC system consisting of:
- 4.14.1 Pump, capable at least of generating binary gradients, pulsation-free, at flows appropriate for the analytical column.
- 4.14.2 Analytical column. Any column which allows for sufficient separation of deoxynivalenol from other interfering components is suitable. Examples are: Phenomenex Octadecylsilane (ODS) 3-Prodigy (15 cm x 4.6 mm i.d.), 5 µm particle size, 100 Å pore size, Octadecylsilane (ODS) 250 mm x 4.6 mm I.D., 3 µm particle size, 80 Å pore size, Octadecyl (C18) 250 mm x 4.6 mm I.D., 5 µm particle size, 180 Å pore size.
- 4.14.3 Pre-column (optional), appropriate for the analytical column used.
- 4.14.4 Auto sampler capable of injecting appropriate volumes with sufficient repeatability.
- 4.14.5 UV detector capable of measuring at 220 nm
- 4.14.6 Data collection system.

4.15 UV spectrophotometer for checking the concentration of the DON stock solution.
4.16 Reservoirs of appropriate size with adaptors to fit the immunoaffinity columns.
4.17 Glass vials, of appropriate size for auto sampler (4.14.4) but with minimum volume of 2.0 ml.
4.18 Syringe filter unit, polyamide (nylon) with 0.45 µm pore size.
4.19 Evaporator, capable of maintaining 50 °C with a steady stream of air or nitrogen.

5. Reagents

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and only double distilled water or water of grade 1 as defined in EN ISO 3696. Solvents should be HPLC grade.

5.1 Acetonitrile.
5.2 Deoxynivalenol (DON), with a minimum purity of 97%.
5.3 Methanol.
5.4 Glacial acetic acid.
5.5 Mobile HPLC-Phase
Mix 15 parts by volume of methanol (5.3) with 84.9 parts by volume of double distilled water and 0.1 parts by volume of glacial acetic acid (5.4). The exact amount of methanol used and whether acetic acid should be used or not depends on the HPLC column chosen for analysis and must be adjusted if necessary. Degas this solution before use.
5.6 Wash Solvent
Mix methanol (5.3) and double distilled water (1:1, v/v).

5.7 DON stock solution: 250 µg deoxynivalenol per ml of acetonitrile

Add 4.0 ml of acetonitrile (5.1) to 5 mg of DON (5.2) for a solution of 1.25 mg/ml. Dilute 1 ml of the 1.25 mg/ml solution to 5.0 ml with acetonitrile for the stock solution of 250 µg/ml. Dilute 200 µl of the 250 µg/ml stock solution in a 2 ml volumetric flask (4.11) with acetonitrile to obtain a diluted stock solution of 25 µg/ml.

To determine the exact concentration record the absorption curve of this 25 µg/ml diluted stock solution with a spectrophotometer (4.15) in the range of 200 to 270 nm in a 1 cm quartz cell with acetonitrile (5.1) as reference. Determine absorption at 220 nm. Calculate mass concentration of deoxynivalenol, ρ_{DON}, in micrograms per millilitre using the following equation:

$$\rho_{DON}\ (\approx 25\ \mu g/ml) = \frac{A_{\max} \cdot M \cdot 100}{\kappa \cdot d}$$

where,

A_{max} = the absorption determined at the maximum of the absorption curve (220 nm),

M = the molar mass of deoxynivalenol (M = 296.3 g/mol),

κ = the molar absorption coefficient of deoxynivalenol in acetonitrile (681 m^2/mol), and

d = the optical path length of the quartz cell in centimeter (1 cm).

Calculate the exact concentration of the 250 µg/ml stock solution using the following equation:

$$\rho_{DON}\ (\approx 250\ \mu g/ml) = \rho_{DON}\ (\approx 25\ \mu g/ml) \cdot 10$$

Stock solution may be stored in the dark for up to 3 months at 44 to 8 °C or at least 6 months at below –18 °C.

5.8 DON spiking solution

Pipette an aliquot of the calibrated DON stock solution (5.7), equivalent to 500 µg DON, into a 5 ml volumetric flask (4.11). Make up to the mark with acetonitrile (5.1). This will result in the spiking solution of 100 µg/ml.

5.9 DON working solution

Pipette an aliquot of the calibrated diluted DON stock solution (5.7), equivalent to 50 µg DON, into a 5 ml volumetric flask (4.11). Make up to the mark with acetonitrile (5.1). This will result in the DON working solution of 10 µg/ml.

5.10 DON Calibration solutions

Calibration solutions are prepared from the 10 µg/ml DON working solution (5.9). For example, add the volumes of 10 µg/ml DON working solution (5.9) shown in Table 8 below into 10 ml volumetric flasks (4.11). Fill the flasks up to the mark with mobile phase (5.5). Deviations are permissible as long as the lowest level is above the limit of detection, the highest level does not lead to saturation of the detector signal, and there are at least two more levels equidistant in-between.

5.11 DON immunoaffinity clean-up columns

The immunoaffinity column contains antibodies raised against DON. The column

TABLE 8
Deoxynivalenol calibration concentrations

Calibration solution	Volume of DON working solution (5.9) [µl]	DON concentration [ng/ml]
1	450	450
2	375	375
3	300	300
4	225	225
5	150	150
6	75	75

should have a capacity of not less than 2500 ng of DON and should give a recovery of not less than 70% when 25 ng of DON are applied in 1 to 2 ml of double distilled water (depending on manufacturer's instructions).

6. Procedure

6.1 Sample Preparation

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Samples should be finely ground and thoroughly mixed using a mill (4.5) and a tumble mixer (4.6) or another process that has been demonstrated to give complete homogenisation before a test portion is removed for analysis.

If the sample has been frozen, allow it to thaw completely before sampling. Mix the sample thoroughly before removing an analytical test portion.

6.2 Extraction

6.2.1 Weigh a 25 g test portion into a 250 ml or 500 ml screw cap flask (4.7).

6.2.2 Add 200 ml of double distilled or deionised water, cap and shake for 1 hour with a shaker (4.3).

6.2.3 Prepare a funnel (4.8) with filter paper (4.9) and filter the extracted sample into a clean 250 ml or 500 ml screw cap flask (4.7).

6.3 Immunoaffinity column clean-up

6.3.1 Attach a reservoir (4.16) to an immunoaffinity column and add 8 ml of deionised or double distilled water.

6.3.2 Transfer 2 ml of the filtered extract (see above; 0.5 ml in case of analytical results above 4000 µg/kg) into the reservoir (4.16). Allow this solution to pass slowly through the column by gravity at a rate of 1–2 drops/second.

6.3.3 When the extract has passed completely through the immunoaffinity column, pass 5 ml of deionised water through the column.

6.3.4 Remove residual liquid by passing nitrogen or air through the column for about 5 seconds. Discard all the eluent from this stage of the clean-up procedure.

6.3.5 Place a HPLC autosampler vial (4.17) under the column and pass 0.5 ml of methanol (5.3) through the column by gravity and collect the eluent. After the

last drops of methanol have passed through the column allow the methanol to remain on the column for approximately 1 minute.

6.3.6 Add another 1 ml of methanol (5.3) and continue to collect the eluent. Carefully pass nitrogen or air through the column in order to collect any residual eluent.

NOTE: Alternatively the manual procedures described for immunoaffinity clean-up (6.3) and elution (6.4) may be performed with an automatic sample preparation unit, provided that volumes and flow rates remain unchanged.

6.4 Preparation of the test solution for HPLC analysis

6.4.1 Place the vial with the eluent in the evaporator (4.19) and carefully evaporate to dryness under nitrogen or air at *ca* 50 °C.

6.4.2 Immediately afterwards cool the HPLC vial to ambient temperature and reconstitute the residue with 0.50 ml of HPLC mobile phase (5.5).

6.4.3 Mix well with vortex mixer (4.4) for at least 30 seconds to ensure the residue is completely re-dissolved. In case of turbidity filter the test solution through a syringe filter unit (4.18).

6.5 Calibration

Prepare a calibration graph at the beginning of every day of analysis by injecting calibration solutions (5.10) at different suitable concentrations onto the chromatograph. Establish the calibration curve prior to analysis of test samples by plotting the concentration of DON [ng/ml] (x-axis) against the peak signal as area or height (y-axis), determine slope and intercept with linear regression, and check the plot using appropriate diagnostics.

6.6 Determination and identification of deoxynivalenol in the test solution

6.6.1 Inject aliquots of the test solutions onto the chromatograph using the same conditions used for preparation of the calibration graph.

6.6.2 Identify the DON peak in the test solution by comparing the retention time with that of the nearest HPLC calibration solution (5.10) injected in the HPLC analysis.

6.6.3 The concentration of deoxynivalenol in the test solution must fall within the calibration range. If the deoxynivalenol level in the test solution exceeds the concentration of the highest calibration solution, the test solution should be diluted with HPLC mobile phase to bring it within the calibration range and re-analysed. The dilution factor must be incorporated into all subsequent calculations.

6.7 HPLC operating conditions

Using the equipment outlined in 4.14 the following conditions have shown to provide adequate separation:

Injection volume: 100–300 µl

UV detection wavelength: 220 nm

Flow rate mobile phase (column): 1.0 ml/minute

If the HPLC pump delivered mobile phase (5.5) through channel A and wash solvent (5.6) through channel B, the gradient profile would look as follows:

TABLE 9
HPLC gradient

Time [min]	Channel A [%]	Channel B [%]
0 to 15	100	0
15 to 25	0	100
25 to 35	100	0

NOTE: Mobile phases prepared with acetonitrile and double distilled or deionised water have also been shown to be suitable alternatives. Such mobile phases may be used provided sufficient separation is achieved.

7. Calculation

Determine from the calibration graph the mass concentration in ng/ml of the deoxynivalenol in the test solution injected into the HPLC column. Calculate the mass fraction of deoxynivalenol, ω_{DON}, in ng/g or µg/kg to one decimal place using the equation below:

$$\omega_{DON} = c_{DON} \cdot \frac{V_3}{V_2} \cdot \frac{V_1}{m_s}$$

where,

c_{DON} = mass concentration of deoxynivalenol as determined using the calibration (6.5);
V_3 = total volume of the test solution (0.5 ml; 6.4.2);
V_2 = volume of the aliquot of the extract used for clean-up (2.0 ml or 0.5 ml; 6.3.2);
V_1 = total volume of the extraction solvent (200 ml; 6.2.2); and
m_s = mass of the extracted test portion (25 g; 6.2.1).

The above equation can be simplified if the described masses and volumes have been used:

$$\omega_{DON} = c_{DON} \cdot 2 \qquad \text{(2 ml of the extracts were cleaned-up)}$$

Should the above calculation render a value above 500 then a new clean-up with 0.5 ml of the sample extract should be prepared (see section 6.3). The simplified equation is then as follows:

$$\omega_{DON} = c_{DON} \cdot 8 \qquad \text{(0.5 ml of the extracts were cleaned-up)}$$

8. Quality Control

8.1 Accuracy

To determine the recovery, spike a deoxynivalenol-free material with the spiking solution (5.8). The spiking level of the working control sample should be within the calibration range (preferably mid-range). The concentration of the solution used should be such that not more than 2 ml is added. Leave the spiked sample to stand for a

minimum of 30 minutes to ensure evaporation of the solvent. Recovery must account for 60 to 120% otherwise the series has to be repeated. This working control should be run with each set and compared to the limits on the established control chart.

8.2 Precision

Inject a mid-range calibration standard as a check after roughly every 5 samples to evaluate the change in peak area. The area should not change more than 10% from the standard peak area in the calibration curve.

9. Remarks

9.1 For the loading capacity of immunoaffinity columns refer to manufacturer's specifications.

9.2 Before using the HPLC procedure, samples can be screened for DON with different test-kits. For example several ELISA-tests are commercially available (see link list in the aflatoxin method). Refer to the manufacturer's product and procedure descriptions.

9.3 The test report shall contain the following data:

- information necessary for identification of the sample (kind of sample, origin of sample, designation);
- a reference to this method;
- the date and type of sampling procedure (if known);
- the date of receipt;
- the date of test;
- the test results and the units in which they have been expressed;
- recovery rate of the working control sample (8);
- a statement whether the results are corrected for recovery;
- particular points observed during the course of the test; and
- operations not specified in the method or regarded as optional, which might have affected the results.

10. References

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DRY MATTER DIGESTIBILITY – *IN VITRO* USING RUMEN LIQUOR

1. Principle

The digestion of feed by the ruminant is simulated *in vitro* in two steps. Firstly, a sample weighed in a test tube is incubated with buffered rumen fluid for 48 hours to remove the digestible carbohydrates. After centrifugation and filtration the feed residue is then incubated with pepsin in hydrochloric acid for another 48 hours to dissolve the digestible

protein. After filtration, the residue is dried, ashed and weighed. The *in vitro* digestibility is finally converted to *in vivo* digestibility level by means of standard samples with known *in vivo* digestibility, run in each sample series. The procedure is based on Tilley and Terry (1963) (see remark 9.3).

2. Scope

The procedure for determination of *in vitro* digestibility with rumen fluid is only applicable to ruminant feeds.

3. Responsibilities

Laboratory Analysts shall perform the analysis as per the described method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Equipment

4.1 Thermos flask.
4.2 Balance, accurate to 0.1 g.
4.3 Analytical balance, accurate to 0.1 mg.
4.4 Centrifuge tubes (plastic) of 75 ml capacity provided with a rubber stop and a gas release valve and a grade mark at 50 ml.
4.5 Centrifuge.
4.6 Water bath with thermostat at 39 ± 1 °C.
4.7 Stirring device.
4.8 Manifolds for centrifuge tubes.
4.9 Filter crucibles or filter paper.
4.10 Drying oven, capable of being maintained at 103 ± 2 °C.
4.11 Muffle furnace, capable of being maintained at 550 ± 20 °C.
4.12 Desiccator.
4.13 pH meter.

5. Reagents

5.1 Ammoniumsulphate, $(NH_4)_2SO_4$.
5.2 Calciumchloride dehydrate, $CaCl_2.2\ H_2O$.
5.3 Carbondioxide gas CO_2.
5.4 Disodiumhydrogenphosphate dodecahydrate, $Na_2HPO_4.12\ H_2O$.
5.5 Hydrochloric acid 1 N.
5.6 Magnesiumchloride hexahydrate, $MgCl_2.6\ H_2O$.
5.7 Pepsin (porc stomach, 766 U/mg).
5.8 Potassiumchloride, KCl.
5.9 Sodiumcarbonate anhydrous, Na_2CO_3.
5.10 Sodiumchloride, NaCl.
5.11 Sodiumhydrogencarbonate, $NaHCO_3$.

6. Procedure

The procedure is preferably carried out according to a fixed time schedule, for example:

Friday

6.1 Weigh into a centrifuge tube (4.4) approximately 0.5 g sample to the nearest 0.1 mg (W1).
With each batch run 4 blanks (centrifuge tubes without sample) and the standard samples.

6.2 Prepare mineral solutions:
Solution A: dissolve 46.5 g $Na_2HPO_4.12\ H_2O$ (5.4), 49.0 g $NaHCO_3$ (5.11), 2.35 g NaCl (5.10) and 2.85 g KCl (5.8) in distilled water and make up to 1 litre with distilled water.
Solution B: dissolve 12.81 g $MgCl_2.6\ H_2O$ (5.6) in 100 ml distilled water.
Solution C: dissolve 5.30 g $CaCl_2.2\ H_2O$ (5.2) in 100 ml distilled water.

Monday starting from about 08:00 hours

6.3 Prepare the buffer solution: for 80–90 samples: mix 800 ml of Solution A, 8 ml of Solution B, 8 ml of Solution C and 12.8 g $(NH_4)_2SO_4$ (5.1) and make up to 4 litres with distilled water. Saturate the solution with CO_2 (5.3) to a pH of 6.9 (control this for approximately 15 minutes with a pH meter (4.13) whilst stirring and warming the solution in the water bath to 39 ± 1 °C (4.6).

6.4 At about 08:00 hours take at least 1.5 litres of rumen fluid from a fistulated cow or 2 fistulated sheep by suction and collect it in a thermos flask (4.1) (previously warmed with water and gassed with CO_2). Transfer immediately to the lab and filter through a double layer of cheese cloth (see remark 9.3).

6.5 Add 1 litre of filtered rumen fluid to the buffer solution (6.3) whilst stirring and gassing with CO_2 (approximately 5 minutes) the whole mixture.

6.6 Add 50 ml of buffered rumen fluid to each centrifuge tube, gas with CO_2, close the tube with rubber stopper and valve and incubate in the water bath at 39 ± 1 °C for 48 hours.

6.7 Shake the tubes by hand at about 13:00 and 17:00 hours.

Tuesday

6.8 Shake the tubes at about 08:00, 13:00 and 17:00 hours.
Wednesday starting at approximately 08:00 hours.

6.9 Make pepsin solution: dissolve 10 g of pepsin (5.7) in 5 litres of water, add 250 ml 1 M HCl (5.5) and warm in a water bath to 39 ± 1 °C (control with thermometer).

6.10 Add 5 ml of a 10% Na_2CO_3 (5.9) solution to each tube and centrifuge (4.5) at 5000 *g* for 3 minutes (keeping the sequence of tubes the same as that at the start of the incubations). **Ensure the tube order is kept the same for all procedures.**

6.11 Filter supernatant through a nylon gauze under suction and add the remainder back to the tube by spraying with the pepsin solution. Add more pepsin solution to make the total volume to the mark 50 ml on the tubes.

6.12 Close the tube with rubber stop and valve and put in the water bath at 39 ± 1 °C for a further 48 hours incubation.

6.13 Shake the tubes by hand at about 13:00 and 17:00 hours.

Thursday

6.14 Shake the tubes at about 0800, 13:00 and 17:00 hours.

6.15 Place the filter crucibles (4.9) in the Muffle furnace at 550 ± 20 °C (4.11) for 2 hours, allow to cool in a desiccator (4.12) for approximately 2 hours, weigh to the nearest 0.1 mg (W2) and put back in the desiccator.

Friday

6.16 Transfer the contents of the tube quantitatively in the crucible and filter under suction.

6.17 Dry the crucibles in the oven at 103 ± 2 °C (about 12:00 hours) until midnight; regulate timer so that oven starts drying again on Monday morning at 02:00 hours.

Monday starting at approximately 08:00 hours.

6.18 Place the crucibles in a desiccator for 2 hours, weigh the crucible containing the residue to the nearest 0.1 mg (W3). Do this also for the blanks.

6.19 Place the crucibles in the Muffle furnace at 550 ± 20 °C for 3 hours, allow to cool in a desiccator for approximately 2 hours, weigh to the nearest 0.1 mg (W4). Do this also for the blanks.

7. Calculation

Calculate percent digestibility of dry matter (% DC of DM) as:

$DC\ of\ DM\ (\%) = [(W1 \times DM\%) - (W3 - W2) - B_{DM}] / (W1 \times DM\%)$

where,

DC of DM = digestibility of dry matter,
W1 = sample weight in g,
DM = DM content of the sample,
W3 = weight of the crucible plus the residue after drying in g,
W2 = weight of the dry crucible in g, and
B_{DM} = average dry matter weight of the blanks.

$DC\ of\ OM\ (\%) = [(W1 \times OM\%) - (W4 - W2) - B_{OM}] / (W1 \times OM\ \%)$

where,

DC of OM = digestibility of organic matter,
W1 = sample weight (g),
OM = OM content of the sample,
W4 = weight of the crucible plus the residue after ashing (g),
W2 = weight of the dry crucible (g), and
B_{OM} = average organic matter weight of the blanks.

Finally, the obtained digestibility values of the samples are converted to *in vivo* digestibility level by using a linear regression equation: $DC_{COR} = DC \times b + a$.

This equation is obtained by regressing the run values (DC_{RUN}) of the standard samples with their reference values (DC_{REF}): $DC_{REF} = a + b\ DC_{RUN}$.

8. Quality Control

In each run at least three reference samples with known *in vivo* digestibility are analysed in triplicate. These reference samples are preferably of a similar nature as the test samples and should cover the digestibility range of the test samples as best as possible. The average run value of the standard samples should not differ by more than 2% from the reference value.

Samples should be analysed at least in duplicate. The difference between duplicates should be lower than 2.5%-units for digestibility values less than 60%, lower than 2.0%-units for values between 60 and 80% and lower than 1.5%-units for values more than 80%.

9. Remarks

9.1 The diet of the donor animals should be standardised. Mostly a hay diet is fed ad libitum. Take the rumen fluid before the morning feed.

9.2 The described procedure gives a measure of the apparent digestibility. A variant of this method where the feed residue after the first stage is treated with neutral detergent solution for 1 hour, gives true digestibility (Van Soest and Robertson, 1985).

9.3 Instead of rumen fluid, which implies the availability and maintenance of rumen-fistulated animals, commercial freeze dried enzyme preparations may be used. These preparations may be pure cellulase or a mixture of polysaccharases. Several procedures are described (Jones and Hayward, 1975; Dowman and Collins, 1982; De Boever *et al.*, 1986). Wherever possible rumen simulated digestion should be used.

10. Interferences and troubleshooting

10.1 It is essential to maintain anaerobic conditions throughout the first stage; thorough gassing of the solutions and the tubes with CO_2 and also attention to the gas release valves that they are working correctly.

10.2 The pH should be carefully controlled and the inoculum be kept as near as possible to 39 °C.

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NIR ANALYSIS

1. Principle

The sample representing the chemical composition of the sample material is measured by NIR spectrometry. Spectral data in the near infrared region are collected and transformed to constituent or parameter concentrations by calibration models developed on representative samples.

2. Scope

The procedure described is applicable for feeds and feed ingredients.

3.Responsibilities

Laboratory Analysts shall perform the analysis as per this method. It is the responsibility of the Laboratory Analyst to ensure that all conditions laid down in the method are met and strictly adhered to. Any deviations from the prescribed method shall be recorded and supervisor notified.

4. Near-infrared (NIR) instruments

NIR instruments based on diffuse reflectance or transmittance measurement in the near infrared wavelength region of 700–2500 nm (14300–4000 cm^{-1}) or segments of this or at selected wavelengths or wave numbers. The optical principle may be dispersive (e.g. grating monochromators) interferometric or nonthermal (e.g. light emitting diodes, laser diodes and lasers). The instrument should be provided with a diagnostic test system for testing photometric noise and reproducibility, wavelength/wave number accuracy and wavelength/wave number precision (for scanning spectrophotometers). The instrument should measure a sufficiently large sample volume or surface to eliminate any significant influence of inhomogeneity derived from chemical composition or physical properties of the test sample. The sample path length (sample thickness) in transmittance measurements should be optimized according to the manufacturer's recommendation with respect to signal intensity for obtaining linearity and maximum signal/noise ratio. In reflectance measurements, a quartz window or other appropriate material to eliminate drying effects should preferably cover the interacting sample surface layer.

5. Sampling

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage.

All laboratory samples should usually be kept under conditions that will not change the composition of the sample from the time of sampling to the time of commencing the procedure.

6. Procedure

6.1 Preparation of test sample

The preparation of samples should be made in the same way as the preparation of the validation samples. It is necessary to apply standard conditions. Before the analysis,

the sample should be taken in such a way as to obtain a sample representative of the material to be analysed.

For specific procedures see specific standards.

6.2 Measurement

Follow instructions for the specific NIR instrument and associated calibrations.

The prepared sample should reach a temperature within the range included in the validation.

6.3 Evaluation of result

For the validation results to be valid, they have to be within the range of the calibration model used.

Results obtained on samples detected as spectral outliers cannot be regarded as reliable.

7. Checking instrument stability

7.1 Control sample

At least one control sample should be measured at least once per day to check instrument hardware stability and to detect any malfunction. Knowledge of the true concentration of the analyte in the control sample is not necessary. The sample material should be stable and, as far as possible, resemble the samples to be analysed. The parameter(s) measured should be stable and, as far as possible identical to or at least biochemically close to the sample analyte. These samples are normally stable for lengthy periods but the stability should be tested in the actual cases. Shifts between control samples should be overlapped to secure uninterrupted control.

The recorded day-to-day variation should be plotted in control charts and investigated for significant patterns or trends.

7.2 Instrument diagnostics

For spectrophotometers the wavelength/wave number accuracy and precision should be checked at least once per week, or more frequently if recommended by the instrument manufacturer, and the results should be compared to specifications and requirements. A similar check of the instrument noise should also be carried out weekly, or at intervals recommended by the manufacturer.

8. Running performance check of calibration

NIR methods should be validated continuously against reference methods to secure steady optimal performance of calibrations and observance of accuracy. The frequency of checking the NIR method should be sufficient to ensure that the method is operating under steady control with respect to systematic and random deviations from the reference method. The frequency depends on the number of samples analysed per day and the rate of changes in sample population.

The running validation should be performed on samples selected randomly from the pool of analysed samples.

It may be necessary to resort to some sampling strategy to ensure a balanced sample distribution over the entire calibration range, e.g. segmentation of concentration range and random selection of test samples within each segment or to ensure that samples with a commercially important range are covered.

The number of samples for the running validation should be sufficient for the statistics used to check the performance. For a solid validation at least 20 samples are needed (to expect a normal distribution of variance). One can fill in the results of the independent validation set for starting the running validation. To continue about 5 to 10 samples every week is quite sufficient to monitor the performance properly. Using less samples it is hard to make the right decision in case one of the results is outside the control limits.

An attempt to force the results within the limits by frequent adjustments of the calibration will not improve the situation in practice. The standard errors of prediction (SEP) should instead be re-evaluated using the latest results.

If the calibration equations after a period of stability begin to move out of control, the calibration should be upgraded. Before this is done, an evaluation should be made of whether the changes could be due to changes in reference analyses, unintended changes in measuring conditions (e.g. caused by a new operator), instrument drift or malfunction etc. In some cases a simple adjustment of the constant term (bias) in the calibration equation may be sufficient. In other cases it may be necessary to run a complete re-calibration procedure, where the complete or a part of the basic calibration set is expanded to include samples from the running validation, and perhaps additional samples selected for this purpose.

Considering that the reference analyses are in statistical control and the measuring conditions and instrument performance are unchanged, significant biases or increased SEP values can be due to changes in the chemical, biological or physical properties of the samples compared to the underlying calibration set.

9. Test report

The test report shall specify:

a) All information necessary for complete identification of the sample;
b) The test method used, with reference to the relevant International Standard;
c) All operating conditions not specified in this International Standard, or regarded as optional;
d) Any circumstances which may have influenced the results;
e) The test result(s) obtained; and
f) The current SEP and bias (if statistically significant), estimated from running a performance test on at least 20 test samples

10. Glossary

General

Reference method. ISO, EN or otherwise internationally recognized and validated method, which gives the "true" or "assigned" value of the parameter to be measured, including the measurement uncertainty.

Indirect method. Method that measures properties that are functionally related to the parameter(s) to be determined. The obtained signal is related to the "true" value(s) as determined by the reference method(s).

NIRS. Near Infrared Spectroscopy

NIR spectroscopy is the measurement of the intensity of the absorption of near-infrared

light by a sample within the range 700–2500 nm (14300–4000 cm^{-1}). NIR instruments use either part of, the whole, or ranges that include this region (e.g. 400–2500 nm).

Multivariate calibration techniques are then used to relate a combination of absorbance values either to composition or to some property of the samples.

NIR. Near Infrared Reflectance NIR spectroscopy where the basic measurements are the absorption of near-infrared light diffusely reflected back from the surface of a sample to be collected by a detector in front of the sample.

NIT. Near Infrared Transmittance NIR spectroscopy where the basic measurements are the absorption of near-infrared light that has travelled through a sample such as a liquid or a solid material (e.g. meat) and is then collected by a detector behind the sample.

NIR network(s). A number of near infrared instruments operated using the same calibration models. Instruments in a network are usually standardized so that the differences in predicted values for a set of standard samples are minimized.

Standardisation (of instruments). A process whereby a group of near infrared instruments are adjusted so that they predict similar values when operating the same calibration model. A number of techniques can be used but these can be broadly defined as either pre-prediction methods where the spectra of samples are adjusted to minimize the differences between the response of a "master" instrument and each instrument in the group and "post prediction" methods where linear regression is used to adjust the predicted values produced by each instrument to make them as similar as possible to those from a "master" instrument.

Z-Score. Performance criterion calculated by dividing the difference between the NIR predicted result and the reference value by a target value for the standard deviation, e.g. the Root Mean Square Error of Prediction (RMSEP) (see last section 'Statistical expressions' of this chapter).

Calibration techniques

Principle Component (PC) Analysis (PCA) is a form of data compression. For a set of samples it works solely with the X (spectral) data and finds Principal Components (factors) according to a rule that says that each PC expresses the maximum variation in the data at any time and is uncorrelated with any other PC. The first PC expresses as much as possible of the variability in the original data. Its effect is then subtracted from the X data and a new PC derived again expressing as much as possible of the variability in the remaining data. You can derive as many PCs as there are either data points in the spectrum or samples in the data set but the major effects in spectra can be shown to be concentrated in the first few PCs and therefore the amount of data that needs to be considered is dramatically reduced. PCA produces two new sets of variables at each stage: PC Scores represent the response of each sample on each PC; PC Loadings represent the relative importance of each data point in the original spectra to the PC. PCA has many uses, e.g. in spectral interpretation, but is most widely used in the identification of spectral outliers.

Principle Component Regression (PCR) uses the scores on each PC as regressors in an Multiple Linear Regression against Y values representing composition of samples. As each PC is orthogonal to every other PC, the scores form an uncorrelated data set with better properties than the original spectra. While it is possible to select a combination of

PCs for regression based on how well each PC correlates to the constituent of interest, most commercial software forces the regression to use all PCs up to the highest PC selected for the model ("the top down approach"). When used in NIRS, the regression coefficients in PC space are usually converted back to a prediction model using all the data points in wavelength space.

Partial Least Square Regression (PLS) is an analytical technique which, like PCA, is a form of data compression. With PLS the rule used to derive the factors is that each factor in turn maximizes the covariance between the Y data and all possible linear combinations of the X data. PLS is therefore a balance between variance and correlation with each factor being influenced by both effects. PLS factors are therefore more directly related to variability in Y values than are Principal Components. PLS produces three new variables, loading weights (which are not orthogonal to each other), loadings, and scores which are both orthogonal. PLS models are produced by regressing PLS scores against Y values. As with PCR, when used in NIRS, the regression coefficients in PLS space are usually converted back to a prediction model using all the data points in wavelength space.

Multiple Linear Regression (MLR) uses a combination of several X variables to predict a single Y variable. IN NIRS, the X values are either absorbance values at selected wavelengths in the near infrared or derived variables such as PCA or PLS scores.

Artificial Neural Networks (ANN) describes a non-linear modelling technique loosely based on the architecture of biological neural systems. The network is initially "trained" by supplying a data set with several X (spectral or derived variables such as PCA scores) values and a reference Y values. During the training process the architecture of the network may be modified and the neurons assigned weighting coefficients for both inputs and outputs to produce the best possible predictions of the parameter values. Neural Networks require a lot of data in training.

Multivariate model. Any model where a number of X values are used to predict one or more Y variables.

Outliers are points in any data set that can be shown statistically to have values that lie well outside the expected distribution. For NIRS data, outliers are normally classified as either X (spectral) outliers or Y (reference data) outliers.

X-outliers are related to the NIR spectrum. An X-outlier can be a spectrum with instrumental faults or from a sample type that is radically different from the other samples or in prediction, a sample type not included in the original calibration set.

Y-outliers are outliers related to errors in reference data, e.g. a transcription error or in the value obtained by the reference laboratory.

Leverage. A measure of how far a sample lies from the centre of the population space defined by a model. Samples with high leverage have high influence on the model. Leverage is calculated by measuring the distance between a projected point and the centre of the model.

Mahalanobis distance. The distance in PC space between a data point and the centre of the PC space (See h-value below). This is a nonlinear measurement. In PC space, a set of samples usually form a curve shaped distribution. The ellipsoid that best represents the set's probability distribution can be estimated by building the covariance matrix of the samples. The Mahalanobis distance is simply the distance of the test point from the centre of mass divided by the width of the ellipsoid in the direction of the test point.

h-value. In some software the Mahalanobis distance is referred to as the "Global h-value" and outlier detection depends upon how many Standard Deviations of h a sample is from the centre. A second measure "Neighbourhood h" is the distance in PC space between a data point and its "n" nearest neighbours and indicates whether a sample is isolated or in a well populated part of the distribution.

Residual(s). The difference between a reference value and the value predicted by a regression model. Used in the calculation of regression statistics.

Test set. When testing a regression model, any set of samples that excludes those used to develop the calibration.

Independent test set. A Test Set that consists of samples that are from a different geographical region, a new plant (in industrial terms), or have been collected at a later time (e.g. from a different harvest) than those used to create and validate a regression model. These samples form a "true" test of a prediction model.

Validation set. Samples used to validate or "prove" a calibration. Usually samples having the same characteristics as those selected for calibration. Often alternate or "nth" samples (ranked in order of the constituent of interest) are allocated to the calibration and validation data sets from the same pool of samples.

Validation samples. See above.

Cross validation. A method of generating prediction statistics where, repeatedly, a subset of samples are removed from a calibration population, a model being calculated on the remaining samples and residuals calculated on the validation subset. When this process has been run a number of times, prediction statistics are calculated on all the residuals.

Full cross validation omits one sample at a time and is run n times (where there are n calibration samples). Where a larger subset is removed, the cross validation cycle is usually run at least eight times before the statistics are calculated. Finally, a model is calculated using all the calibration samples. Cross Validation should be used with caution. Firstly, cross validation statistics tend to be optimistic when compared with those for an independent test set. Secondly, care should be taken if there is any duplication in the calibration data (e.g. the same sample scanned on several instruments or at different times) to always assign all copies of the same sample to the same cross validation segment otherwise very optimistic statistics are produced.

Overfitting. The addition of two many regression terms in a multiple linear regression. As a result, when samples not in the calibration set are predicted, statistics such as RMSEP or SEP are much poorer than expected.

PLS factors. See PLS above.

Scores/score plots. Plots where the scores on one PC or PLS factor are plotted against those of another PC or PLS factor. Most useful if sample ID or concentration values are used to identify each point in the plot. Patterns in the data can then be seen which are not obvious from the raw data.

Statistical expressions

Bias. The difference between the mean reference value and the mean value predicted by the NIR model.

SEC. For any calibration model, the Standard Error of Calibration (SEC) is an expression of the average difference between predicted and reference values for samples used to

derive the model. In this and subsequent statistics this expression of the average difference refers to the square root of the sum of squared residual values divided by the number of values corrected for degrees of freedom, i.e. the limit that covers 68% of the errors. This is necessary as some residuals are positive, others negative.

SECV. For a calibration model, the Standard Error of Cross Validation (SECV) is an expression of the bias corrected average difference between predicted and reference values for the subset of samples selected as prediction samples during the cross validation process (see ''Cross Validation" above).

SEP. The Standard Error of Prediction (SEP) is an expression of the bias corrected average difference between predicted and reference values predicted by a regression model when applied to a set of samples not included in the derivation of the model.

RMSEP. The Root Mean Square Error of Prediction is an expression of the average difference between reference values and those predicted by a regression model when applied to a set of samples not included in the derivation of the model. NOTE: RMSEP includes any bias in the predictions.

RMSECV. The Root Mean Square Error of Cross Validation (RMSECV) is an expression of the average difference between predicted and reference values for the subset of samples selected as prediction samples during the cross validation process (see "Cross Validation" above). *NOTE:* RMSECV includes any bias in the predictions.

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Every sector of the livestock industry, the associated services and the wellbeing of both animals and humans are influenced by animal feeding. The availability of accurate, reliable and reproducible analytical data is imperative for proper feed formulation. Only reliable analysis can lead to the generation of sound scientific data.

This document gives a comprehensive account of good laboratory practices, quality assurance procedures and examples of standard operating procedures as used in individual specialist laboratories. The adoption of these practices and procedures will assist laboratories in acquiring the recognition of competence required for certification or accreditation and will also enhance the quality of the data reported by feed analysis laboratories. In addition, ensuring good laboratory practices presented in the document will enhance the safety of the laboratory workers. The document will be useful for laboratory analysts, laboratory managers, research students and teachers and it is hoped that it will enable workers in animal industry, including the aquaculture industry, to appreciate the importance of proven reliable data and the associated quality assurance approaches. An additional effect of implementing and adopting these approaches will be strengthening of the research and education capabilities of students graduating from R&D institutions and promotion of a better trading environment between developing and developed economies. This will have long-term benefits and will promote investment in both feed industries and R&D institutions.

ISBN 978-92-5-107050-5 ISSN 1810-1119

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I2441E/1/11.11