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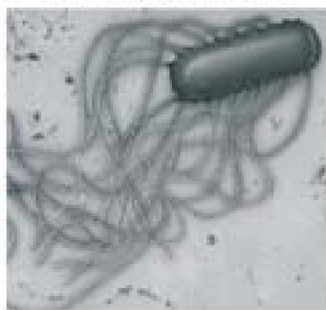

paper

# ASSESSING QUALITY AND SAFETY OF ANIMAL FEEDS

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# ASSESSING QUALITY AND SAFETY OF ANIMAL FEEDS

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
Rome, 2004

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# FOREWORD

Feeds and forages are variable in composition. Feed analysis provides information for farmers to optimize nutrient utilization in animal feeds; for feed compounders to prepare feed mixtures suitable for different animal production systems; for researchers to relate animal performance to feed characteristics; and for plant breeders to optimize the nutritive value of new varieties. Also of concern are the undesirable contaminants of animal feeds which may have a direct bearing on the safety of foods of animal origin. This book brings together six reviews on these subjects from the FAO Electronic Journal AGRIPPA in printed form.

The keynote article by Irene Mueller-Harvey describes current procedures for feed analysis and procedures to improve standards. She describes how to achieve quality control, quality assurance, laboratory accreditation and proficiency testing. Standard and widely accepted methods are described together with recent developments in feed analysis. Topics covered include: sample preparation, analysis of major components (dry matter, ash and minerals, crude protein, fat, fibres and starch) and of secondary plant products (tannins, mycotoxins and other contaminants). Developments in the analysis of whole samples by near infrared reflectance spectroscopy are mentioned and the potential of this technique to by-pass traditional feed analysis by directly predicting animal responses.

Feedstuffs vary because of genetic differences and as a consequence of feed processing. The paper by Gizzi and Givens considers the importance of these factors for the compound feed manufacturer, the farmer and the policy maker. Data variability also results from differences in the methodologies used to obtain the information. Chemical analysis procedures and animal study protocols may vary according to the laboratory or institute involved. Understanding the variation in chemical and nutritional characteristics of feedstuffs is vital to the effective use of feed information in livestock production.

The paper by Harinder Makkar describes the potential of the *in vitro* gas production method for evaluating nutritional quality of feed resources for ruminants. This technique enables selection of a feed or feed constituent for high efficiency of microbial protein synthesis in the rumen along with high dry matter digestibility, and provides a basis for development of feeding strategies to maximize efficiency. In addition, this technique provides an experimental tool to study the effects of various natural and synthetic compounds and their adverse or beneficial effects on rumen fermentation.

Felix D'Mello covers the microbiology of animal feeds, including forages, cereal grains, oilseed by-products and compound feeds. He notes the beneficial effects of lactic acid bacteria in the fermentation of forages during the process of ensilage. Lactic acid bacteria and yeast cultures have also been attributed with beneficial properties as feed probiotics for reducing scouring and increasing growth performance in farm animals. Animal feeds may become contaminated with harmful bacteria such as *Salmonella, Listeria* and *E. coli*. Cereal grains and oilseed by-products are regularly contaminated with fungi occurring as plant pathogens or developing during storage. Major adverse effects arise in farm animals due to the production of mycotoxins by certain species and strains of these fungi. He discusses potential methods for reducing the prevalence of deleterious fungi and regulations to control these feed contaminants, particularly mycotoxins.

In a second article, Dr D'Mello reviews the range of contaminants and toxins arising from anthropogenic and natural sources. He considers the distribution of heavy metals, radionuclides, mycotoxins, plant toxins, antibiotics and microbial pathogens in cereals, complete feeds and forages, together with the impact on farm livestock productivity and on the safety of resulting products. He considers methods of avoiding contamination and the regional significance of controls and legislation.

The final paper by Peter Hughes and John Heritage explores the developing controversy surrounding the use of antibiotics as growth

promoters for food animals. These drugs are used at low doses in animal feeds and are considered to improve the quality of the product, with a lower percentage of fat and higher protein content in the meat. They may also help to control zoonotic pathogens such as *Salmonella, Campylobacter, Escherichia coli* and enterococci. Use of antibiotics, particularly at low doses, is associated with selection for resistance in pathogenic bacteria and it has been argued that the use of antibiotic growth-promoters may result in bacteria resistant to antibiotics used in clinical or veterinary practice, thus compromising the continued use of antimicrobial chemotherapy. The paper reviews the use of antibiotics as growth promoters and examines some of the alternative methods for achieving meat of high quality.

This publication intends to provide most recent information on the impact of animal feeds on food quality, food safety and the environment and to thus improve the basis for managing such risks which are increasingly at the centre of public and individual consumer attention.

Further articles will be published from time to time and can be read on-line at: http://www.fao.org/agrippa/ . Peer reviewed and edited documents are published in the AGRIPPA system and immediately available to readers. Subjects covered include livestock production, animal nutrition and feeding, and farming systems.

**Samuel Jutzi**
Director
FAO Animal Production and Health Division

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADF | acid detergent fibre |
| ATP | Adenosin-Three Phosphate |
| AAFCO | American Association of Feed Control Officials |
| AOAC | Association of Official Agricultural Chemists |
| BSE | bovine spongiform encephalopathy |
| CE | capillary electrophoresis |
| CF | crude fibre |
| CP | crude protein |
| CT | condensed tannins |
| CRM | certified reference materials |
| DAS | diacetoxyscirpenol |
| DDE | dichlorodiphenyldichloroethylene |
| DDT | dichlorodiphenyltrichloroethane |
| DM | dry matter |
| DOM | digestibility of organic matter |
| DON | deoxynivalenol |
| E-As | enzyme-linked antibodies |
| EE | ether extract |
| ELISA | enzyme linked immunosorbent assay |
| EU | European Union |
| FAPAS | Food Analysis Performance Assessment Scheme |
| FDA | Food and Drug Administration |
| GATT | General Agreement on Tariffs and Trade |
| GE | gross energy |

| | |
|---|---|
| GMO | genetically modified organisms |
| HPLC | high performance chromatography |
| HT | hydrolysable tannins |
| LAB | lactic acid bacteria |
| MALDI-TOF MS | matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| ME | metabolizable energy |
| MIC | minimum inhibition concentration |
| mMBM | mammalian meat and bone meal |
| MRSA | methicillin-resistant *Staphylococcus aureus* |
| MS | mass spectrometry |
| NDF | neutral detergent fibre |
| NFE | nitrogen free extractive |
| NIR | near infrared reflectance spectroscopy |
| NIV | nivalenol |
| NOAH | National Office of Animal Health |
| OM | organic matter |
| OP | organophosphorus pesticides |
| OTA | Ochratoxin A |
| PCBs | polychlorinated biphenyls |
| PCR | Polymerase Chain Reaction |
| PDA | potato dextrose agar |
| PEG | polyethylene glycol |
| PTS | proficiency testing schemes |
| SAC | Scottish Agricultural College |
| SCFA | short cahin fatty acid |
| SMCO | S-methyl cysteine sulphoxide |

| | |
|---|---|
| VAM | Valid Analytical Measurement |
| VFA | volatile fatty acids |
| TLC | thin layer chromatography |
| TSE | transmissable spongiform encephalopathyes |
| vCDJ | variant Creutzfeldt-Jakob disease |
| VTEC | Vero-Toxigenic *Escherichia coli* |
| ZEN | zearalenone |
| WHO | World Health Organization |

# Modern techniques for feed analysis

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This review describes accepted procedures for feed analysis with particular emphasis on rapid analyses. The analysis of individual chemical compounds tends to be easier to standardize than some less well-defined, but nutritionally important parameters, e.g. oil, fibre, starch. Inter-laboratory comparisons have demonstrated unacceptably large variation. A coordinated effort is required to reach consensus procedures amongst feed analysts using quality control measures. Unfortunately, there are few certified reference materials (CRMs) and proficiency schemes for animal feeds. In cases of complex mixtures of pro- or anti-nutrients, e.g. tannins, structure-function relationships have not yet been elucidated and, therefore, no particular assay can be recommended to plant breeders. Finally, the concept of 'traceability of analytical measurements' has been translated to 'traceability of feeds' in the form of a 'Feed Passport'. This will become increasingly important following the meat-and-bone meal issue in cattle feed and new soybean varieties that led to the bovine spongiform encephalopathy (BSE) crisis and the genetically modified organisms (GMOs) disputes.

## 1. INTRODUCTION

Most forage and by-product feeds are variable in composition. Only very few, such as dried whole whey, are uniform in composition and do not usually require analysis (Schingoethe, 1991). Feed analysis provides information for:

- farmers to optimize nutrient utilization in animal feeds;
- feed compounders to prepare feed mixtures suitable for different animal production systems;

- researchers to relate animal performance to feed characteristics; and
- plant breeders to optimize the nutritive value of new varieties (Madsen *et al*. 1997; Wrigley, 1999).

Many rapid analytical tests have been developed over the last few years and are of use when faced with a need for quick decisions or when confronted by large numbers of samples, e.g. at entry points of shipments, trading situations, on contamination sites or in plant breeding programmes.

This review will cover the main issues which prompted developments in quality assurance and control of analysis. Information will also be given on accreditation of laboratories, together with useful addresses. Subsequent sections will describe standard and widely accepted methods, highlight areas that require particular attention and refer to recent developments in feed analysis. Topics covered will include: sample preparation, analysis of major components (dry matter, ash and minerals, crude protein, fat, fibers and starch) and of secondary plant products (tannins, mycotoxins and other contaminants). Developments in the analysis of whole samples by near infrared reflectance spectroscopy will be mentioned and the potential of this technique to by-pass traditional feed analysis by directly predicting animal responses.

The reader may consult the following books for general information on feedstuff evaluation and diet formulation in relation to animal production: Wiseman and Cole (1990), Minson (1990), AFRC (1993), Fahey *et al.* (1994), Givens *et al.* (2000a). Detailed protocols for analytical methods can be found in MAFF (1986), Watson (1994), AOAC (1995) and on the web (Appendix 1).

This review will only briefly mention a few chromatographic and mass spectrometric techniques in relation to analysis of secondary products. It will not cover the analysis of whole samples by new *in vitro* digestibility techniques, pyrolysis-mass spectrometry, nuclear magnetic resonance or other spectroscopic techniques as these have been covered elsewhere (Mauricio *et al.*, 1999; Givens *et al.* 2000a).

# 2. QUALITY CONTROL, ASSURANCE, ACCREDITATION AND PROFICIENCY TESTING IN FEED ANALYSIS

## 2.1 Inter-laboratory variation

The need for agreement on methods to obtain comparable, useful data became obvious with the beginning of 'scientific agriculture' in the 19th century (Midkiff, 1984). Different methods led to widely varying results and to regulatory confusion. In 1884, the first meeting of the Association of Official Agricultural Chemists (AOAC) began to tackle fertilizer analysis and soon, 1886, included feedstuff testing. The AOAC president stated in 1896 'The matter of the analysis of foods and feedstuffs, as shown by the experience of the association, is one of the most difficult questions connected with the work of this organization'. The AOAC was renamed in 1965 to 'Association of Official Analytical Chemists' to take cognizance of the fact that the AOAC now had a wider scope (Midkiff, 1984).

A report in 1985 on the quality of data relating to Pb and Cd analysis in food laboratories concluded that results were inaccurate and the validity of the data was in doubt (Patey, 1996). Similar conclusions have been reached for a wide range of data relating to feed analysis. It would appear that feed analysis is somewhat lagging behind food analysis. Selected examples are given below, in order to illustrate the magnitude of the problem.

Bailey and Henderson (1990) concluded that there was an urgent need to improve oil and sugar determinations, since these methods had relatively poor precision amongst 15 feed laboratories. These data are commercially important as they are used when making labeling declarations and for energy content estimation. Lanari *et al.* (1991) commissioned a study of brewer's grain, dried beet pulp, lucerne and hay with 20 feed analysis laboratories. Unacceptably large coefficients of variation were found for mean oil content (determined as ether extract): 3.5 percent (cv = 17.8); lignin: 5.4 percent (cv = 27.3); neutral detergent fibre (NDF) 55.6 percent (cv = 7.4 percent). Coefficients of variation were 5 percent or less for dry matter (DM), crude protein (CP), crude fibre (CF), ash, acid detergent fibre (ADF),

and gross energy (GE). Beever *et al.* (1996) submitted two contrasting maize silages to 10 commercial feed laboratories. They concluded that current feedstuff analysis provided unacceptable variation and required national standards, as coefficients of variation were 12.7 percent for CP and 16 percent for starch. In addition, Givens *et al.* (2000b) also list several more references reporting large inter-laboratory variation for several analyses relevant in feed evaluation: organic matter (OM) digestibility, *in situ* rumen degradation, gas production kinetics, *in vitro* digestibility, metabolizable energy (ME) and GE.

*In vitro* and *in vivo* digestibility measurements have been shown to suffer from similar inter-laboratory variation. Madsen and Hvelplund (1994) sent five feeds to 23 laboratories in 17 countries and found "differences ... in protein degradabilities between laboratories too large to be acceptable" when using nylon bags in the rumen of cows or sheep. The within laboratory variation, however, of protein degradation was acceptable. They concluded that sample preparation and processing and the bags themselves varied considerably between laboratories and made detailed recommendations for the nylon bag procedure. Authors also recommend that a 'standard feed is made available for all laboratories for routine checking of analytical procedure'. A small ring test of the Tilley-Terry method between 3 laboratories suggested that it was a robust method for OM digestibility of roughages (Madsen *et al.*, 1997). However, problems were encountered with concentrates and highlights that careful standardization is required. Not surprisingly, feed manufacturers have moved to an enzyme method and such results are expected to be more reproducible (Madsen *et al.*, 1997).

There are large differences between the different *in vitro* digestibility techniques (rumen fluid versus cellulase based techniques) which are used to predict *in vivo* digestibility (De Boever *et al.*, 1994; Aufrère and Michalet-Doreau, 1988). Clearly, such differences in methods need to be resolved.

These data collectively support the introductory statement that feed analysis can be difficult. Strict adherence to method details is important, especially if the method itself defines the component, e.g.

crude fat, NDF, lignin, as empirical fractions are chemically not well defined (Bailey and Henderson, 1990). Zeeman and Bonn (1995) have also suggested that there should be an international definition of starch. However, large variation may highlight problems in analytical methods and thus encourage closer investigation. Problems with too high NDF values, for example, are likely to be stem from incomplete solubilization of starch. Recently, Thiex *et al.* (1996) investigated the observed large variation in reported vitamin A results, which was noted by the American Association of Feed Control Officials (AAFCO) who operate a feed check sample programme. Several recommendations were made to reduce errors of Vitamin A in animal feed and pet food analysis.

## 2.2 The benefits of quality assurance programmes

A review of the literature for methods of feed grain analysis concluded that it was not possible to assess if variation in reported values was due to genotypic, environmental factors or inter-laboratory differences. Petterson *et al.* (1999), therefore, recommended the use of quality assurance schemes, inter-laboratory evaluation programmes and reference materials.

The Global Environmental Monitoring Scheme of the World Health Organization (WHO) tested the performance of European Union (EU) laboratories that contribute data on food contamination (Weigert *et al.*, 1997). This involved five proficiency tests, 136 laboratories in 21 countries using their own preferred methods for the analysis of trace metals (Pb, Cd, Hg in milk powder), pesticides (organochlorine, organophosphorus, pyrethroid in spinach powder), nitrate in spinach powder, aflatoxins in nut-based animal feeds. Only 60 percent reported accurate for trace metals, 41 percent for pesticides, 43 percent for nitrate, 88 percent for aflatoxins and 53 percent for patulin (average = 68 percent).

Key *et al.* (1997) summarized the results of the UK Food Analysis Performance Assessment Scheme (FAPAS) from 1990-1996. For pig feeds (moisture, ash, oil, protein, fibre, Cu), only 76 percent of laboratories achieved satisfactory results and for nutritional analysis

80 percent were satisfactory. The FAPAS study also reveals that some analyses are more difficult than others. 91 percent of aflatoxin data were satisfactory, 81 percent of veterinary drug residues, 86 percent of dichlorodiphenyldichloroethylene (DDE), but only 71 percent of dichlorodiphenyltrichloroethane (DDT) and 65 percent of Ca data. Surprisingly, Ca analysis has shown little improvement over the years. Inter-laboratory comparisons and proficiency testing can highlight inappropriate methods, e.g. analysis of veterinary drug residues using certain immunological tests. However, once laboratories were participating in proficiency tests on a regular basis (e.g. FAPAS) the average percentage for accurate results increased.

Horwitz (1993) observed "most experimentation dealing with analytical methodology in biological sciences has been conducted within a single laboratory. Method validation by other laboratories was considered not only unnecessary but also detrimental because, in the words of one commentator, 'the results are too variable'. Within the last two decades, however, it has become increasingly apparent that a collaborative inter-laboratory study is the only way to estimate the variability characteristics of methods...." and to meet the increasing demand by regulatory programmes for high quality data.

## 2.3 How to achieve valid data

The UK Department for Trade and Industry launched an initiative in 1994 on Valid Analytical Measurement (VAM) incorporating four main principles.

### *2.3.1 Principle 1: Measurements should be made using properly validated methods*

Properly validated methods will provide information on the performance of an analytical technique, such as accuracy and precision, ruggedness, operating range, selectivity and limits of detection. It is essential when reporting a measured value to also give its uncertainty. Otherwise, it is not possible for users of the data to know what confidence to place in the data. It needs to be recognized that problems of communication can arise from the word

'uncertainty', as a layperson may misinterpret the statistical term 'error' into 'inaccurate data'! (Williams, 1996).

Guidelines are now available for the statistical evaluation of analytical tests and laboratory performance (Bailey and Henderson, 1990): the inter-laboratory precisions are generally a function of concentration. Horwitz' group found that the within-laboratory variation was approximately one-half to two-thirds of the between-laboratory variation and can be used as a 'bench mark for judging previously unevaluated methods' (Bailey and Henderson, 1990).

### *2.3.2 Principle 2: Quality assurance protocols should incorporate certified reference materials (CRMs) to ensure traceability of measurements*

CRMs are used for:

- Calibration and verification of measurements under routine analysis conditions
- Internal quality control and quality assurance schemes
- Verification of the correct application of standardized methods
- Development and validation of new methods of measurement.

Unfortunately, only few CRMs are available for validation of analytical methods for feed analysis, especially for proximate analysis (Crosby, 1995). However, CRMs exist for minerals in two animal feeds: hay powder (Ca, K, Mg, P, S, Zn, I, N, Kjeldahl-N) and rye grass (As, B, Cd, Cu, Hg, Mn, Mo, Ni, Pb, Sb, Se, Zn) (Maier *et al.*, 1990; see Appendix 1 for CRM suppliers). Information on the in-house production of reference materials can be found in Walker and Brookman (1998). It is good practice to include external CRMs or in-house reference materials into all analytical procedures.

### *2.3.3 Principle 3: Laboratories should seek an independent assessment of their performance for particular tests, preferably by participating in national and international proficiency testing schemes (PTS)*

PTS independently assess the performance of analytical laboratories. The true concentration of an analysis can be determined by addition

of a known amount of analyses to a base material or better still through the use of a consensus value produced by a group of analysts. Guidelines exist for organizing a collaborative study to evaluate analytical methods (AOAC, 1988). Appendix 1 lists several proficiency testing schemes.

### *2.3.4 Principle 4: Laboratories should seek independent approval of their quality assurance arrangements, preferably by accreditation or licensing to a recognized quality standard.*

It should be recognized that only certain accreditation schemes are appropriate for laboratories performing chemical analysis, e.g. NAMAS M10, ISO 90025, ISO/IEC 17025, EN 4501. General guidelines have been written on how to prepare for accreditation (EURACHEM/WELAC, 1998). Gangaiya and Morrison (1992) listed general problems of setting up quality assurance in some developing country laboratories. Appendix 1 lists several accreditation organizations.

## 3. SAMPLE PREPARATION

Good analytical data require that samples be representative of the whole and that their integrity has been ensured during transport to the laboratory and during their preparation (drying and grinding). Relevant guidelines for feeds can be found in Feeding Stuffs (1988), AOAC (1995), Crosby (1995) and Wrigley (1999). Drying may adversely affect the analysis of sugars, vitamins, certain trace elements (F, Se, B), and ammonia and volatile fatty acids (VFAs) in silages (MAFF, 1986). Caution needs to be taken when grinding lupin and chickpea seeds (Petterson *et al.*, 1999).

## 4. MOISTURE

Several methods exist to determine moisture content of feeds: oven drying at 105° C for 16h, 125° C for 4h or 135° C for 3h (AOAC, 1995). Molasses should be dried at 70° C because of high levels of volatile compounds (Petterson *et al.*, 1999). Oven-drying is problematic with silages and high fat feeds; vacuum-oven drying at 95-100° C (AOAC,

1995) or at <70° C, Karl Fischer or toluene distillation are alternative techniques (Crosby, 1995; Cherney, 2000). Baker *et al.* (1994) found significant variation in moisture contents between laboratories, which were tracked down to variable temperature gradients in ovens (Givens, pers. communication).

## 5. ASH

Crude ash is determined either by ashing at 600° C for 2 h or between 500-550° C for 12-16 h (Midkiff, 1984; Petterson *et al.*, 1999). For difficult samples, Crosby (1995) lists some special techniques. Flameless atomic absorption spectrometry on solid feed samples for trace levels of Cu represents an interesting development (Anzano *et al.*, 1994). However, sample size at present is restricted to 2-4 mg. This technique, therefore, requires further development for general applicability in feed analysis.

## 6. CRUDE PROTEIN

Crude protein data are standard for evaluating the protein value of forages (Cherney, 2000). The historic developments and critical points in Kjeldahl nitrogen measurements (choice of catalyst, temperature and digestion times) have been summarized by Lakin (1978) and Midkiff (1984). The Dumas technique represents an alternative method for total nitrogen. Total nitrogen is determined after combustion of the sample and several commercial instruments are now available for this. The measurements are rapid taking only 2-5 minutes per sample. The small sample size (20 to 500 mg) is the key problem with the Dumas method. Samples should be finely ground and preferably dry to avoid too frequent changes of costly reagents. Therefore, the best method for some materials, e.g. silages, is still the Kjeldahl procedure as drying would remove ammonia-N.

The need for corrosive and toxic reagents is the main disadvantages of the Kjeldahl method.

Total Dumas nitrogen values can be slightly higher than the Kjeldahl values as Dumas N also includes nitrate and organic compounds that are highly resistant to acid digestion (Lakin, 1978;

Petterson *et al.*, 1999). As a result, exceptional differences can occur with some biological matrices, e.g. fruits, vegetables and fish, with Kjeldahl-N:Dumas-N ratios as low as 0.15 (Simonne *et al.*, 1997).

A factor of 6.25 is used to convert total nitrogen in animal feeds into crude protein. However, the amino acid composition varies between foods and therefore different factors have been suggested to convert total N to crude protein (Petterson *et al.*, 1999). It can range from 5.14 for grains and oilseeds to 6.38 for dairy products. As there is some doubt about the universal validity of such factors, authors should report crude protein values together with the factors used in their calculation (Lakin, 1978).

It should be noted that none of the dye-binding methods were universally acceptable between laboratories for protein determinations (Petterson *et al.*, 1999). Recently, Strong and Duarte (1992) described a simple and rapid biuret method for protein determination in wheat, rice and soybeans. This has been applied to a wide range of different grains and only requires a blender, reagents, colorimeter at 550 nm and minimum operator training. The method requires initial calibration against protein values obtained by other techniques. This procedure may be of interest to grain and feed merchants as it can be completed in five minutes.

## 7. FIBRE ANALYSIS

It is important to recognize that all fibre determination employs 'empirical' methods, i.e. the method determines the final result and any deviation from the analytical protocol will produce a different result (Midkiff, 1984; Crosby, 1995). Midkiff (1984) summarized the history of crude fibre analysis. Cherney (2000) contrasted the Weende proximate analysis system, which originated in 19th century and has hardly changed since then, with the Van Soest system developed in the 1960s. He cautions against the use of CF, nitrogen free extractives (NFE), ether extract (EE) in feed evaluation systems, as they do not sufficiently separate digestible from non-digestible fractions. The Van Soest system is now widely used for forage evaluation as it provides

useful measurements for nutritionally important parameters, such as structural carbohydrates (Goering and Van Soest, 1970; AOAC method 973.18).

Several groups have since modified the original manual method (Cherney *et al.*, 1989; Van Soest *et al.*, 1991) and Chai and Uden (1998) have described a simple oven-based procedure. The micro-NDF method by Pell and Schofield (1993) is another modification, which allows NDF to be determined on small sample sizes (10 to 50 mg). Moore and Hatfield (1994) comprehensively reviewed the composition and analytical methods for structural and non-structural carbohydrates citing research involving both ruminants and monogastric animals. Mertens (1997) concluded, "the only fibre method that can be used on all types of feeds is the method recommended by the National Forage Testing Association" (Undersander *et al.*, 1993, Hintz and Mertens, 1996; see also Appendix 1 for Web site).

Starch removal from NDF can be difficult and requires pre-treatment with α-amylase either overnight (McQueen and Nicholson, 1979) or by using heat-stable α-amylase (Sigma product A3306) during the last 30 minutes of NDF extraction and during filtration (Cherney *et al.*, 1989). Difficult samples may also require pretreatment with 8M urea (Van Soest *et al.*, 1991). Filtration problems may be due to incomplete starch removal. However, errors in fibre analysis may also stem from aged crucibles, as crucibles can be damaged at too high temperatures or by rapid rates of heating or cooling in muffle ovens (Crosby, 1995).

Fibretec (Tecator, Hoenganaes, Sweden) and the recent FibreAnalyzer (ANKOM Technology Co., Fairport, NY, USA) are instruments for NDF and ADF extractions. Studies indicated that ADF by Fibretec and FibreAnalyzer are comparable for most feeds. However, in our experience NDF in starch containing feeds is best analysed by the FibreAnalyzer as it removes starch more efficiently (unpublished data).

## 8. STARCH

Starch consists essentially of two components, amylose and amylopectin. Amylose polymers contain up to 2000 glucose units connected through linear 1-->4 linkages. Amylopectin is a highly branched polymer containing 2,000 to 220,000 glucose units with 1-->4 and 1-->6 linkages. Therefore proper extraction techniques are crucial for successful starch analysis (Petterson *et al.*, 1999).

Starch is determined by pre-treating with 80 percent ethanol in boiling water to remove low molecular weight sugars, followed by gelatinization and solubilization, before extracting and hydrolyzing the starch (Faichney and White, 1983; Åman and Graham, 1990; Hall, 1997). Incomplete dissolution and incomplete accessibility to enzymes tend to be the main problems in starch analysis. McCleary *et al.* (1994; 1997) reported that a ringtest involving 29 laboratories to evaluate the Megazyme enzyme kit for cereal products and some animal feeds produced good agreement for total starch content. This enzyme kit was accepted as an AOAC method (996.11) (Petterson *et al.*, 1999).

The proportion of amylose and amylopectin depends on the source of the starch. Starch digestibility is not necessarily related to total starch content as the amylose: amylopectin ratio and processing affect the extent of starch digestion (Allen *et al.*, 1997; Reynolds *et al.*, 1997). The Rapid Visco Analyser measures pasting properties of starch in grain and Wrigley (1999) speculated that this may be related to starch digestibility, but this awaits further research.

## 9. CRUDE FAT

Fat in plant-derived feeds consists mainly of mono-, di- and triacylglycerides, free fatty acids and phospholipids. Feeds also often contain fats from animal and other waste products. Depending on the rendering processes used, heating or storing can lead to unsaponifiable matter, oxidized and polymerized fatty acids which will contribute to crude fat values, but not be of nutritional value (Edmunds, 1990). Midkiff (1984) described the history of crude fat analysis and the sample types that caused problems.

Several official methods exist to determine crude fat in animal feeds (AOAC 945.16 and 920.38; MAFF 1986). Crude fat methods are empirical methods and procedural details must be closely adhered to. They are based on solvent extractions with or without hydrolysis. Crude fat is extracted by the EU procedure A with petroleum ether (40-60° C) and the dried residue weighed (Feeding Stuffs 1988). Acid hydrolysis is used as a pretreatment in EU procedure B. However, it is recommended that animal feeds are first extracted with petroleum ether (40-60° C) (Procedure A) and then subjected to acid hydrolysis, before re-extraction with petroleum ether (40-60° C) (Procedure B). This minimizes losses during filtration of the acid digest, which can occur if protected fats, i.e. Ca and Mg salts of fatty acids are present (Edmunds, 1990; Crosby, 1995).

Neither procedure A nor B will give satisfactory results if feeds contain milk products. The Rose-Gottlieb method is required for such feeds, as the alkaline pretreatment frees occluded lipids from protein capsules (ISO 1211:1984). Similarly, canned dog foods require extraction with a series of solvents after hydrolysis (Budde, 1952; Midkiff, 1984; AOAC 954.02).

Problems may arise if diethyl ether is used instead of petroleum ether as suggested in some methods. Slightly higher values may result if water has not been removed completely from either the sample or the solvent, as some compounds, such as urea and sugars, are slightly soluble in diethyl ether in the presence of small amounts of water (Midkiff, 1984; AOAC 920.39).

Great care is required when performing solvent extractions. Solvent are usually recycled between a lower electric heating source and an upper water-cooling system in a Soxhlet apparatus. Accidents are not uncommon with such a set-up. For this reason, a different type of extractor has been developed recently (Brown and Mueller-Harvey, 1999). The Soxflo instrument requires neither heat nor cooling water and is based on a dry-column procedure. The sample is packed into a column and the extracting solvent drips slowly through it. Crude fat values determined by the Soxflo and Soxhlet procedures were found to be in close agreement. If sample drying and packing

procedures are evaluated against the Soxhlet procedure, good and reliable crude fat data can be obtained in 60 to 90 minutes.

## 10. SECONDARY PLANT PRODUCTS: TANNINS

Although a whole range of different plant secondary products exists, only tannins will be discussed here. The main reason for this is the persistent confusion that surrounds tannins in animal nutrition. Analytical techniques have often been misapplied and data misinterpreted. However, reference will be made to rapid analyses of mycotoxins and other secondary plant products which can contaminate or affected the nutritive value of animal feeds (see Section 11).

Tannins comprise a diverse group of phenolic compounds, varying in molecular size from 500 to possibly 28,000 Daltons (Jones *et al.*, 1976; Mueller-Harvey and McAllan, 1992; Schofield *et al.*, 2001). Some are easily extracted by aqueous solvents, others are not and can be measured as fiber or protein bound tannins (Jackson *et al.*, 1996; Reed, 1986). The naming and thereby classification of condensed (CT) or hydrolysable tannins (HT) is somewhat misleading as some of the 'condensed' tannins are relatively easily degraded oxidatively, whereas some of the 'hydrolysable' tannins will resist all attempts at hydrolysis (Mueller-Harvey, 1999; 2001).

Negative and positive animal responses have been attributed to tannins, i.e. ranging from animal death to increased growth rates (Butter *et al.*, 1999; Mueller-Harvey, 1999). Despite many animal studies involving tanniniferous feeds, little attempt has been made to elucidate the relationship between animal production and tannin structures. Progress is unlikely to be achieved by the continued use of colorimetric tannin assays alone (Petterson *et al.*, 1999). Lowry *et al.* (1996) put it succinctly: "the simplicity of absorbance measurements masks the problems of extracting meaningful data".

A general reagent produces varying colour yields from different phenolic compounds, which includes tannins (Folin and Ciocalteau, 1927). However, phenolics and tannins tend to occur in mixtures and quantitation is not possible unless isolated standards are used, which

are representative of the exact composition of the material being examined. Procedures for colorimetric methods for CT and HT have been described by Graham (1992), Waterman and Mole (1994) and Hagerman (Web site).

Porter *et al.* (1986) recommended a modification of the butanol-HCl assay. This assay is excellent for detecting the presence of CT. However, for quantization purposes it should only used if isolated tannins are used as calibration standards as tannin structures have a marked effect on colour yield (Giner-Chavez *et al.*, 1997). Even within the same species, there can be sufficient structural variation to warrant isolated tannin standards from different accessions in quantitative work (Stewart *et al.*, 1999). More recently, a CT assay based on acidified 4-dimethylamino-cinnamaldehyde (DMACA-HCl) was described (Li *et al.*, 1996). This assay proved more sensitive than the vanillin-HCl method, which can suffer from interference by water (Terrill *et al.*, 1990). The DMACA assay was also used successfully on thin layer chromatography (TLC) plates and as a histochemical assay. This method has not yet been examined thoroughly against interferences from monomeric flavanols.

The analysis of HT has been reviewed by Mueller-Harvey (2001). Again, assays are best used to detect HT, as accurate quantitation would require isolated standards (Hagerman *et al.*, 1997). Furthermore, there are just a few known species that produce only gallotannins, from which gallic acid can be hydrolysed before detection (Willis and Allen, 1998). More usually, gallo- and ellagitannins occur together as mixtures. Ellagitannins are much more difficult to measure (Wilson and Hagerman, 1990) although the free ellagic acid can be measured by high performance chromatography (HPLC) (Mueller-Harvey *et al.*, 1987). However, as stated above, many other so-called HT does not release gallic or ellagic acid.

It is recommended that additional assays be used in tannin analysis to overcome some of the problems of the colorimetric tests, e.g. the Yb-precipitation method (Reed *et al.*, 1985; Giner-Chavez *et al.*, 1997; Krueger *et al.*, 2000a) and/or one of the protein or polymer binding assays (Makkar *et al.*, 1987; Dawra *et al.*, 1988; Makkar *et al.*, 1993)

when screening novel fodder plants. If facilities are available to measure radioactivity, tannins can also be measured by binding to 125I labelled BSA (Hagerman and Butler, 1980) or 14C-polyethylene glycol (PEG) (Silanikove *et al.*, 1996). Tannins can be estimated directly in feed samples, without prior extraction in the latter method.

The Yb-method precipitates total phenolics (i.e. tannins, flavonoids and other phenolics) which correlate positively with butanol-HCl CT (Reed *et al.*, 2000). The advantage is that phenolics are determined by gravimetry without the need for standards. Interestingly, the amount of PEG bound per gram of sample correlated highly significantly with *in vitro* N digestibility and appeared to be a more meaningful measurement than the colorimetric asssays (Jones and Palmer, 2000). Correlations with CT measurements by vanillin-HCl were better than by butanol-HCl.

In view of the problems of colorimetric assay, it seems surprising that TLC has not been used more widely. TLC reveals the presence of CT vs HT, low vs high molecular weights of CT, the subclasses of CT or HT (procyanidins or prodelphinidins, gallo- or ellagitannins), plus semiquantitative information based on colour intensity (Mueller-Harvey *et al.*, 1987). Tannins from different species have also been compared by reverse phase HPLC (Mueller-Harvey *et al.*, 1987), size exclusion chromatography (Yanagida *et al.*, 1999; Hedqvist *et al.*, 2000) and normal phase chromatography (Tanaka *et al.,* 1984; Okuda *et al.*, 1989; Hagerman *et al.*, 1992). Okuda *et al.* (1989) reviewed the mass spectrometric analysis of tannins. More recently, ESI-MS (Guyot *et al.*, 1997; Marais *et al.*, 2000) was used to determine tannin molecular weights. New developments in MALDI-TOF mass spectrometry (Hedqvist *et al.*, 2000; Krueger *et al.*, 2000a and b) succeeded in determining the molecular weights and composition of complex tannin mixtures.

## 11. IMMUNOASSAY TECHNIQUES: FOR MYCOTOXIN AND OTHER FEED CONTAMINANTS

Mycotoxins pose severe hazards to humans and animals. Mycotoxins in feeds, along with many other organic compounds, can be analysed

by either instrumental [HPCL, gas chromatography (GC), capillary electrophoresis (CE)] or biochemical (immunoassays) techniques.

Skerritt and Appels (1995) described the basic principles of enzyme-linked immunoasorbent assays (ELISAs) and illustrated the different forms of techniques (direct or indirect assays, sandwich or competition assays). For example, in the direct competitive ELISA format, enzyme-linked antibodies (E-As) coat the surface of a well plate or test tube, which are supplied in a test kit. The operator extracts the feed sample and places the extract onto the surface. Compounds of interest, i.e. antigens, bind to the E-As thereby releasing them from the surface. E-As are then washed away and an enzyme substrate is added to the remaining, bound E-As. This procedure results in an inverse relationship between antigen concentration and colour production: the higher the antigen concentration, the more E-As have been washed away and the smaller the colour yield of the reaction.

Several commercial kits are available for aflatoxins, zearalenone and other mycotoxins, alkaloids, glucosinolates, insecticides, herbicides, fungicides, various environmental pollutants, vitamins in foods and animal feeds. Morgan (1995) reviewed these ELISA techniques and listed suppliers' addresses. Schneider *et al.* (1991) developed an interesting dipstick technique for the simultaneous detection of several mycotoxins.

ELISAs can be used by regulatory authorities, quality control laboratories and in research laboratories. They require relatively little user training and can be used in small laboratories or under field conditions. Low cost and high speed make ELISAs ideal for on-site monitoring of stored grain and for assessing rapidly if the maximum residue levels of traded animal feeds have been exceeded. They can also be used for checking suspected spillages. High sample throughput facilitates elimination of large numbers of negative, uncontaminated samples. It is, however, recommended that samples giving positive results for a contaminant should be re-analysed by conventional instrumental techniques to ensure absence of matrix interference and accuracy of the data (Petterson *et al.*, 1999).

Some commercial test kits compare well with standard AOAC methods for aflatoxins in animal feeds (Trucksess *et al.*, 1989 and 1990; Cochrane, 1991) and can be used for quantitative or semiquantitative measurements in as little as 3 minutes. Several of these have now obtained AOAC approval (Trucksess *et al.,* 1989). ELISAs can be tailored to be selective for individual compounds or compound classes, parent compounds or metabolites and even for isomers. Highly sensitive ELISAs for M1 in milk samples have been reported (Kawamura *et al.*, 1994). Of interest in the current European BSE crisis is a report by von Holst *et al.* (2000), who evaluated the applicability of a commercial ELISA method to detect proper heat treatment of pork and beef meals.

**A word of caution**. ELISA procedures – like any analytical method – need to be carefully evaluated as some solvents used for analyte extraction may adversely affect antibody performance (Morgan, 1995). In an inter-laboratory study, ELISA tests gave good results for ZON, but accuracy with the DON kits was poor (Schuhmacher *et al.*, 1997). Matthews *et al.* (1996) investigated commercial kits for three organophosphorus pesticides (OP) for laboratory and field use. The authors found good correlation with GC methods (r-values between 88 and 98 percent). However, as the protocols supplied with the kits were somewhat confusing, the authors provided improved protocols for use by grainstore keepers, millers and malsters. It was also found that the operating ranges of the kits were not as wide as claimed by the manufacturer. In addition, some cross-reactivity of the antibodies was observed with structurally related compounds. Nonetheless, the study showed that ELISA tests could be used reliably to measure OP residues on stored grain.

The most common problems encountered in ELISAs are incomplete washing steps, pipetting problems, insufficient temperature control of reagents and plates, degradation of conjugate (may inhibit good binding to antibody) or loss of enzyme activity (Gee *et al.*, 1995). Problems with sample matrices are also common and can be detected by comparison of standards in an uncontaminated

or ‘blank’ matrix. Recovery studies of standard additions may show up matrix problems. Alternatively, a dilution curve with sample matrix solution can be compared against a standard calibration curve. If the slopes are not parallel, a matrix effect is likely.

Finally, when purchasing an assay kit, the following criteria should be considered: price per assay, sensitivity, cross-reactivity, suitability for the chosen matrix, availability of published validation by independent workers and technical support (Gee *et al.*, 1995).

## 12. NEAR INFRARED REFLECTANCE SPECTROSCOPY (NIRS) FOR WHOLE SAMPLE ANALYSIS

Deaville and Flinn (2000) wrote a brief and clear introduction to the basic principles of NIRS. An accessible and more detailed review of applications and a description of the mathematical treatments of NIR spectra has been given by Shenk and Westerhaus (1995). Givens and Deaville (1999) have also reviewed NIRS in relation to feed analysis and animal nutrition.

The advantages of NIRS (Givens and Deaville, 1999) over traditional techniques are:

- Rapid, as minimal or no sample preparation necessary
- On-the-spot analysis of the whole sample, i.e. a non-destructive technique, allows simultaneous measurement of several parameters
- High precision
- High throughput makes NIR a cheap technique on a sample basis
- Environmentally friendly: no reagents, no chemical waste

The limitations of NIRS are:

- Suitable for major feed components, not for minor components
- Great care needed in developing calibrations as these are matrix specific
- Complexity in choice of data treatment is confusing for the novice

- Calibration procedures are time consuming and only worthwhile for subsequent analysis of large sample numbers
- High instrument costs

NIRS was developed in the 1950s and 1960s for quantitative analysis and applied to feed analysis in the 1970s (Norris *et al.*, 1976). By the late 1970s, NIRS was routinely used for protein measurements in grain. The NIRS region covers wavelengths between 730 and 2500 nm and is the infrared region that is particularly suited to quantitative analysis (Givens and Deaville, 1999). The main absorption bands of water are at 1940 and 1450 nm, of aliphatic C-H bonds at 2310, 1725, 1400 and 1210 nm, of O-H bonds around 2100 and 1600 nm and N-H bonds at 2180 and 2055 nm. Spectra are depicted in the form of the reciprocal log of reflectance (log1/R) and provide little direct information. Various components in the feed matrix produce a series of overlapping bands, which results in a smooth, rolling line. However, first- and second-order derivatives of the log1/R spectra can be used to resolve these overlapping bands.

NIRS data are generally subjected to a mathematical pretreatment to reduce interferences from light scatter (Barnes *et al.*, 1989). Then one of several different multivariate calibration methods is used to relate the spectral data from a sufficiently large and representative sample set to the primary, 'wet chemistry', data (Blanco *et al.*, 1997). Finally, calibrations are subjected to validation procedures with an independent set of samples. A simple monitoring procedure has been developed to minimize NIRS analysis errors (Shenk *et al.*, 1989).

Great care should be taken in developing NIR calibrations (references in Deaville and Flinn, 2000). Calibrations should be based on at least 50 samples, but often many more are required (>150). Givens and Deaville (1999) pointed out that 'NIRS is largely a secondary technique requiring calibration using samples of known composition determined by using standard methods (primary techniques)'. A problem can arise, when the primary methods do not define well the chemical constituent, e.g. drying at 100°C to determine moisture does not necessarily define water content or 6.25

x total N does not necessarily describe protein content adequately (Shenk and Westerhaus, 1995).

It has been stressed that consistent sample preparation is required, as variations in particle size, residual moisture content and packing density can adversely affect NIR spectra. However, new developments of NIR software, such as the noise repeatability file, have succeeded in reducing the sensitivity to residual sample moisture (Baker *et al.*, 1994; Shenk and Westerhaus, 1995). Alternatively, by using a coarse transport cell, a larger surface area of fresh grass silage can be screened, thus eliminating the need for dry silages (Park *et al.*, 1999a, b).

NIRS has been accepted as an official AOAC method for crude protein and ADF (AOAC 989.03) and for moisture (AOAC 991.01; Barton and Windham, 1998). It has also been used for determining starch and non-starch polysaccharides, fat and oil, metabolizable energy, insect or weed seed contamination in feed grains (Wrigley, 1999) and for the analysis of dried forages (Murray, 1993). It can be used to identify feeds and perform authenticity checks (De Boever *et al.*, 1993). In addition, heat damaged protein, fungal contamination and adulteration can be detected with modern pattern recognition software (Givens and Deaville, 1999).

It is also possible to transfer calibrations developed on an expensive scanning instrument to cheaper filter instruments in a network (Puigdomènech *et al.*, 1997). This ISI cloning software also allowed a successful transfer of NIR calibrations developed for fresh grass silages from a Foss to a Bran & Luebbe instrument (Park *et al.* 1999b).

Researchers are now aiming to predict directly the functional properties of feeds to animals, i.e. nutrient supply and production responses such as live weight gain, milk fat and protein or meat composition, rather than measuring feed components (Wrigley, 1999). Good predictions have been achieved for organic matter digestibility *in vivo* (Barber *et al.*, 1990); ME content (Givens *et al.*, 1992) and voluntary feed intake (see references in Deaville and

Flinn, 2000). It has also been possible to predict nutritionally relevant products, such as lactic acid, VFAs and cumulative gas volumes with calibrations based on 800 fresh silages (Deaville and Flinn, 2000). The ultimate aim is to formulate diets for optimum animal productivity, cost effectiveness and the least environmental effects (Givens and Deaville, 1999).

## 13. CONCLUSIONS

This review describes general procedures for feed analysis and includes recent developments towards faster techniques for fibre (FibreAnalyzer) and fat (Soxflo) analysis, new enzyme kits for rapid starch analysis, ELISAs for mycotoxins and other feed contaminants and progress in whole feed analysis by NIR spectroscopy.

It would appear that feedstuff analysis is difficult in comparison with other analyses (Midkiff, 1984; Key *et al.* 1997). The analysis of an individual, well-defined chemical compound tends to be easier to standardize between laboratories than some of the less well-defined, but nutritionally important parameters, e.g. oil, fibre, starch or digestibility of animal feeds. A better understanding of these parameters would help to improve their measurement.

Several inter-laboratory comparisons have demonstrated that variation tends to be unacceptably large, and that a coordinated effort has to be made to reach consensus procedures amongst feed analysis laboratories. Significant progress has been achieved through international efforts in producing CRMs for minerals, pesticides and pollutants in a range of environmental and food matrices. However, there are fewer CRMs for feeds, with still no CRM for NDF, and a dearth of proficiency schemes for animal feeds.

Plant breeders have access to several assays that determine levels of single, simple constituents responsible for beneficial or harmful effects (amino acids, n-3 fatty acids, minerals, mycotoxins, glucosinolates, alkaloids) (Wrigley 1999).

However, in cases of more complex mixtures of pro- or anti-nutrients, e.g. starches, tannins, lectins, structure-function

relationships have not yet been elucidated and, therefore, no particular assay can be recommended for use in breeding programmes (Caygill and Mueller-Harvey, 1999). Similarly, tests for biologically active compounds, e.g. phytoestrogens or antioxidants, could be used more widely by plant breeders once the beneficial target compounds have been clearly identified.

Finally, the concept of good analytical practice, that ensures 'traceability of measurements' through use of validated methods and CRMs, has also been translated to 'traceability of feeds' by a feed manufacturer in the form of a 'Feed Passport'. This is likely to become increasingly important following recent experience with meat and bone meal in cattle feed and new soyabean varieties that led to the BSE crisis and GM disputes.

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# Appendix 1

## 1. USEFUL WEBSITES FOR FURTHER INFORMATION (TEACHING AND TRAINING)

http://www.lgc.co.uk/best/terp/terpoindex.htm
http://www.agric.rdg.ac.uk/Facilities/FAL/FAL_frameset.htm
'Accreditation and Quality Assurance' - a journal for quality, comparability and reliability in chemical measurement.

## 2. WEB SITES FOR ANALYTICAL METHODS

Methods for forage testing:
http://www.dfrc.wisc.edu/foragetesting_nfta.html
Methods for tannin analysis: http://miavx1.muohio.edu/~hagermae/

### 2.3 Addresses and Web sites for Certified Reference Materials

National Institute of Standards & Technology
Standard Reference Materials Programme
Building 202, Room 204
Gaithersburg, MD 20878-9950, USA
Fax: +1 301 948 3730

Community Bureau of Reference
Commission of the European Communities
Rue de la Loi 200
B-1049 Brussels, Belgium
Fax: +32 2 295 8072

The Office of Reference Materials
Laboratory of the Government Chemist
Queens Road
Teddington

Middlesex TW11 0LY, U.K.
e-mail: orm@lgc.co.uk
http://www.lgc.co.uk

Promochem Ltd
6 South Mundells
Haslemere Industrial Estate
Welwyn Garden City
Herts AL7 1EP, U.K.
Fax: +44 1707 396 677

Breitlaender Eichproben und Labormaterial GmbH
Postfach 8046
4700 Hamm 3, Germany
Fax: +49 2381 403 189

National Institute for Environmental Studies
Japan Environment Agency
P O 16-2 Onogawa
Tsukuba
Ibaraki 305, Japan
Fax: (0298) 51 6111

## 2.4 Addresses for RING TESTS or Proficiency Schemes

National Forage Testing Association, USA:
http://www.dfrc.wisc.edu/foragetesting_nfta.html

Dr V Houba
Department of Soil Science and Plant Nutrition
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6700 EC Wageningen/ The Netherlands
Fax: +31 8370 83766

Dr A. Eijgenraam
Bedrijfslaboratorium voor Grond- en
Gewasonderzoek
Postbus 115
6860 Oosterbeek, The Netherlands
Fax: 0031 26 333 7831
(Please note: two feed samples per year for
non-commercial laboratories only)

ALASA
P O Box 14105
Verwoerdburg 0140
Republic of South Africa
Fax: +27 12 664 1431

Dr A.L. Patey
FAPAS Secretariat
Ministry of Agriculture, Fisheries and Food
Food Science Laboratory
Norwich Research Park
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Norfolk NR4 7UQ, U.K.
Fax: +44 1603 501 123

### 2.5 Web sites for Accreditation

http://www.european-accreditation.org
http://www.aoac.org/techprog/Intro98.htm
http://www.ukas.com/
http://www.info.gov.hk/itc/eng/quality/hkas/hkas.shtml